# EPITOPE CONSTRUCTS COMPRISING ANTIGEN PRESENTING CELL TARGETING MECHANISMS

## FIELD OF THE INVENTION

The present invention relates to products and methods to elicit a targeted immune response. In particular, the invention relates to constructs that carry antigen presenting cell - targeted epitope sequences.

## BACKGROUND OF THE INVENTION

Cancer remains the second leading cause of death in the United States. Non-surgical therapy for breast, lung, and colon, as well as many other solid tumors, is presently poor.

### Tumor-Associated Antigens

Tumor-associated antigens (TAAs) are important biochemical markers of tumor cells and include, for example, mutated cellular proteins such as mutated tumor suppressor gene products, oncogene products (including fusion proteins), and foreign proteins such as viral gene products. Non-mutated cellular proteins may also be TAAs if they are expressed aberrantly (*e.g.*, in an inappropriate subcellular compartment) or in supernormal quantities. Given the numerous steps of cellular transformation and sometimes bizarre genotypes observed in cancer cells, it could be argued that tumor cells are likely to contain many new antigens potentially recognizable by the immune system. It also has been reported that different tumors of related tissue or cellular origin may share the same TAA (Sahasrabudhe *et al.*, 1993, J. Immunol., 151:6302-6310;

Shamamian *et al.*, 1994, Cancer Immunol. Immunother., 39:73-83; Cox *et al.*, 1994, Science 264:716; Peoples *et al.*, 1993, J. Immunol., 151:5481-5491; Jerome *et al.*, 1991, Cancer Res., 51:2908-2916; Morioke *et al.*, 1994, J. Immunol., 153:5650-5658). These data support the possibility that specific anti-tumor immunotherapies targeting TAAs, such as vaccines, may be active against more than one form of cancer.

## Immunotherapy of Cancer

In cancer, tumor-specific T cells that are capable of binding to and lysing tumor cells displaying the corresponding tumor-associated epitopes or antigens on their cell surfaces can be derived from patients. Tumor-specific T cells are localized at several sites within cancer patients, including in the blood (where they can be found in the peripheral and mononuclear cell fractions), in primary and secondary lymphoid tissue, *i.e.*, the spleen, in ascites fluid in ovarian cancer patients (tumor-associated lymphocytes or "TALS"), or within the tumor itself (tumor infiltrating lymphocytes or "TILS"). Of these T cell populations, TILs have been the most useful in the identification of tumor antigens and epitopes thereof.

The specificity of tumor-specific T cells is based on the ability of the T cell receptor (TCR) to recognize and bind to a short amino acid sequence that is presented on the surface of the tumor cells by MHC class I and, in some cell types, class II molecules. In brief, these amino acid binding sequences (also termed "TAA ligands" or "TAA epitopes") are derived from the proteolytic degradation of intracellular proteins encoded by genes that are either uniquely or aberrantly expressed in tumor cells.

TAAs presented in the context of major histocompatability antigen (MHC) class I complexes on either the tumor cell itself or on antigen-presenting cells (APCs) are capable of inducing tumor-specific cytotoxic T lymphocytes (CTLs). The presence of co-stimulatory molecules, such as B7-1 and B7-2, on APCs and the secretion of IL-2 promote the differentiation of recruited CD8+ lymphocytes into CTLs.

Indeed, there is substantial evidence indicating that the immune system plays a critical role in the prevention of cancer and the control of tumor growth. This includes the occasional observation of spontaneous tumor regression, the correlation of spontaneous regressions with the presence of tumor-infiltrating lymphocytes (TILs) and

the identification of TILs that are specific for TAAs. However, as evidenced by the incidence rates of cancer, the immune response is often not sufficient to successfully combat the tumor.

The growth and metastatic spread of tumors, to a large extent, depend on their capacity to evade host immune surveillance and to overcome host defenses. All tumors express antigens that are recognized to a variable extent by the immune system, but in many cases an inadequate immune response is elicited because of partial antigen masking or ineffective activation of effector cells. Tumor escape from immune effectors is most often caused by weak immunogenicity of TAAs, antigen masking, or overall immunosuppression, a characteristic of advanced cancer. Failure of antigen processing or binding to MHC molecules, inadequate or low-affinity binding of MHC complexes to T-cell receptors, or inadequate expression of co-stimulatory adhesion molecules in conjunction with the antigen-presenting MHC complex may all lead to poor immunogenicity of TAAs and impaired antitumor response.

In recent years, there has been a renewed interest in the development of cancer vaccines. This has resulted, in part, from the identification of new TAAs and an increased understanding of the importance of antigen presentation and lymphocyte activation. Immunotherapeutic strategies are now accepted as superior in terms of the specificity that they offer in targeting only tumor cells as opposed to the existent chemotherapy or radiation therapy that is more general and invasive with many associated side effects.

Immunotherapeutic strategies have been developed that attempt to "modulate" various aspects of the immune response associated with cancer. Vaccination with immunogenic peptides, administration of *in vitro* expanded and activated immune effector cells, *in vivo* effector cell expansion with cytokine therapies, or genetic modification of either immune effectors or tumor cells with cytokine genes or genes encoding co-stimulatory molecules were shown to activate anti-tumor immune responses.

The literature reveals that neither adoptive transfer of tumor-specific CTLs nor specific active immunotherapy with whole tumor cells or cell-derived preparations leads to eradication of tumors or long-term survival in more than a minority of patients. In contrast, it has been demonstrated *in vitro* that peptides have succeeded in priming T

cells where cell-derived preparations have failed. It has been therefore suggested that epitopes recognized by multiple CTL lines would be promising candidates for use in peptide-based anti-tumor vaccines.

Importantly, however, while administration of synthetic peptides derived from TAAs elicited tumor-specific immune responses *in vitro*, attempts to provoke anti-tumor responses *in vivo* by vaccination with TAA protein or peptide fragments have often been unsuccessful, presumably because these protein or peptide fragments failed to access the cytosol of a cell and, therefore were not properly processed and presented to effector cells.

Taken together with the fact that TAA peptides generally bind weakly to the MHC molecules, the data described above suggest that improving MHC binding affinity and intracellular targeting of TAA peptide immunogens should improve their immunogenicity.

Several new prospects for preparation of T cell vaccines have been suggested based on the identification and characterization of MHC-associated peptides. Synthetic peptides that correspond to these T cell epitopes may represent ideal subunits for safe vaccines. However, synthetic peptides are poor immunogens because of their small molecular size and very short serum half-life. To circumvent these disadvantages, a variety of recombinant proteins that carry short immunogenic epitopes have been described (Leclerc *et al.*, Int. Rev. Immunol. 11: 123-132 (1994); Freimuth and Steinman, Res. Microbiol. 141: 995-1001 (1990); Evans *et al.*, Nature 339: 385-388 (1989); Bona *et al.*, Chem. Immunol. 65: 179-206 (1997); Baier *et al.*, J. Virol. 69: 2357-2365 (1995)). For example, genetically engineered antibody molecules can function as such delivery systems for T cell peptides and have the advantage of being self-proteins devoid of the side effects sometimes associated with microbial vaccines and, moreover, have a long half-life compared with synthetic peptides (Billetta *et al.*, Proc. Natl. Acad. Sci. USA 88: 4713-4717 (1991); Zaghouani *et al.*, Science 259: 224-227 (1993); Rasmussen *et al.*, Proc. Natl. Acad. Sci. USA 2001 98:10296-301).

## TAA Identification and Modification

The development of peptide-based anti-tumor immunotherapies is further

hindered by the absence of a reliable iterative method to identify TAAs. Current protocols involve isolating and assaying extremely pure MHC molecules from antigen presenting cells (APCs) (Chicz and Urban, 1994, Immunol. Today, 15:155-160) or the determination of structural features of the MHC/peptide complexes using X-ray crystallography (Meng and Butterfield, Pharm Res 19:926-32 (2002)). Conventional methods for culturing and subcloning of tumor-specific T cells are known in the art. Once a potent anti-tumor T cell population is recovered, it can be used to identify tumor antigens via conventional, but often tedious, expression cloning methodology (Kawakami *et al.* (1994) Proc. Natl. Acad. Sci. USA 91:3515-3519), or assaying epitopes produced in phage libraries (Scott and Smith, 1990, Science 249:386-390; Cwirla *et al.*, 1990, Proc. Natl. Acad. Sci., 87:6378-6382; Devlin *et al.*, 1990, Science, 249:404-406).

In a different approach, requiring a known pathogen- or tumor-related antigen, methods that attempt to identify the native epitope have been developed. For example, putative epitopes can be predicted using a computer to scan the sequence of the gene (antigen) for amino acid sequences that contain a "motif" or a defined pattern of amino acid residues associated with a particular MHC (HLA) allele. See, *e.g.*, Englehard (1994) Annu. Rev. Immunol. 12:181; Rammenesee *et al.* (1993) Annu. Rev. Immunol. 11:213. The "predicted" epitope sequences can then be synthesized and tested. Although many epitope sequences have been "predicted" from scanning full-length protein sequences by "motif", upon testing in standard functional assays, the vast majority of these "predicted" epitopes failed to be immunogenic. Other techniques include, for example, peptide elution followed by database searching (Hunt *et al.* (1992) Science 255:1261; Udaka *et al.* (1992) Cell 69:989); isolation and identification of the antigen from complex antigen mixtures (Van de Wal *et al.* (1998) Proc. Natl. Acad. Sci. USA 95:10050; Lamb *et al.* (1987) Immunology 60:1); screening expression libraries and subsequent database searches (Boon *et al.* (1994) Annu. Rev. Immunol. 12:337; Neophytou *et al.* (1996) Proc. Natl. Acad. Sci. USA 93:2014; Gavin *et al.* (1994) Eur. J. Immunol. 24:2124); peptide positional scanning of combinatorial libraries (Gundlach *et al.* (1996) J. Immunol. Meth. 192:149; Blake *et al.* (1996) J. Exp. Med. 184:121; Hiemstra *et al.* (1997) Proc. Natl. Acad. Sci. USA 94:10313; Hemmer *et al.* (1997) J. Exp. Med. 185:165 1), and the like.

More recently, certain pathogen- and tumor-related proteins have been immunologically mimicked with synthetic peptides whose amino acid sequence corresponds to that of an antigenic determinant domain of the pathogen- or TAA. Despite these advances, peptide immunogens based on native sequences generally perform less than optimally with respect to inducing an immune response. Thus, a need exists for modified synthetic antigenic peptide epitopes with enhanced immunomodulatory properties.

Combinatorial peptide and non-peptide chemistry methodologies have provided additional tools for determining T cell epitopes. Epitopes so determined typically "mimic" the native epitope in that they bear a definable sequence similarity thereto (*e.g.*, conservative substitutions as well as identical amino acids), but not necessarily absolute identity therewith. Epitope mimics can be designed by directly modifying the sequence of known epitopes or defined de novo with randomized molecular libraries followed by database searching to identify the native antigen. (Gavin *et al.* (1994) Eur. J. Immunol. 24:2124; Blake *et al.* (1996) J. Exp. Med. 184:121; Chen *et al.* (1996) J. Immunol. 157:3783; Strausbauch *et al.* (1998) Intl. Immunol. 10:421; Valmori *et al.* (2000) J. Immunol 164(2):1125-1131; Needels *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:10700-4; Ohlmeyer *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926; Van der Zee, 1989, Eur. J. Immunol., 19:43-47; International Patent Publication No. WO 92/00252).

Thus, there is a need in the art to identify effective methods to target tumor cells for immune clearance by generating an effective anti-tumor immune response. In particular, there is a need in the art to develop new peptide-based immunogens which would induce a strong targeted anti-tumor immune response. As outlined above, in connection with the need for new improved peptide-based immunogens, there is also a need to develop new modified synthetic antigenic peptides with enhanced immunomodulatory and targeting properties over peptides derived from native tumor-associated antigens (TAAs).

The present invention addresses these and other needs in the art with the discovery of an effective therapy based on the identification of non-native epitopes

derived from cancer antigens, and their assembly into epitope string constructs, in conjunction with targeted delivery of these epitope string constructs to appropriate antigen presenting cells (APCs), as well as the inclusion of sequences in the constructs to direct appropriate processing and presentation of the incorporated epitopes within the APCs.

## SUMMARY OF THE INVENTION

The present invention provides a peptide construct comprising at least one epitope sequence, wherein the construct also includes or is associated with an antigen presenting cell (APC) targeting mechanism and is capable of stimulating an immune response such as, for example, a cytotoxic T-cell and/or helper T-cell and/or B-cell response.

The epitope contained in the peptide construct of the present invention can be, for example, a CD8+ T cell epitope, a CD4+ T cell epitope, a B cell epitope, or any combination thereof. As disclosed herein, the epitope can be derived from any antigen. For example, the epitope can be derived from a cancer antigen (also termed tumor-associated antigen or TAA), a viral antigen, a bacterial antigen, a protozoan antigen, or a fungal antigen. Preferably, the epitope is derived from a TAA. In one of the embodiments, the TAA is a carcinoembryonic antigen (CEA).

Preferably, the epitope is a non-native epitope, which binds with high affinity to the MHC molecules and is useful for modulating immune responses to the corresponding native epitope from which this non-native epitope is derived. The non-native epitope sequences of the present invention preferably differ from their natural counterparts in that they contain alterations in amino acid sequence, relative to the native sequence, in the MHC binding domain, thereby conferring tighter binding to the MHC molecule. Alternatively or in addition, they may contain mutations in the putative T cell receptor-binding domain, resulting in an increased affinity for the T cell receptor (TCR). These differences from the native sequence confer advantages in the methods of the present invention over the use of the native sequence, in that the non-native epitopes of the invention have enhanced immunomodulatory properties.

Also preferably, the peptide construct of the invention is a polyepitope construct comprising more than one epitope, wherein said epitopes are derived from one or more different antigens. In polyepitope constructs of the invention, at least one epitope is preferably a cytotoxic T cell (CTL) epitope. In a specific embodiment, the construct represents an epitope string with epitopes arranged consecutively.

Also preferably, each of the epitopes in the peptide construct of the invention is flanked on at least one side, and preferably on both sides, by a spacer (flanking) sequence comprising an internal processing sequence. The internal processing sequence is designed to facilitate the endosomal and/or lysosomal processing of the construct once it has reached the antigen presenting cell (APC).

The APC targeting mechanism is comprised of (i) an APC targeting sequence, which directs the construct to antigen presenting cells (APCs) within the subject and, optionally (ii) a vehicle, such as, *e.g.*, a microsphere or liposome, that also acts as a targeting mechanism and/or preserves the viability of the construct until it has reached its intended APC and/or mediates the controlled release of the construct. The APC targeting sequence may be covalently or non-covalently attached to the epitope. Preferably, the APC targeting sequence is covalently attached to the N- terminus or C-terminus of the sequence flanking respectively the first or the last epitope in the epitope string. In a specific embodiment, the APC targeting sequence is derived from a sequence capable of mediating interaction with such cell surface proteins as, for example, Heat Shock Protein Receptor (HSR) such as CD91, C-type lectin receptors such as Mannose Receptor (MR), DEC-205 and DC-SIGN, and IgG Fc receptor (FcR) such as Fc$\gamma$RI.

In a specific embodiment, the peptide construct of the invention may be represented by the following general formula:

**N-[APC targeting sequence]-[flanking sequence with internal processing sequence]-[epitope 1]-[flanking sequence with internal processing sequence]-[epitope 2]...[epitope n]-C**

or

**N-[epitope 1]-[flanking sequence with internal processing sequence]-[epitope 2]...[epitope n]-[flanking sequence with internal processing sequence]-[APC targeting sequence]-C**

wherein n is the number of epitopes in the construct and wherein the internal processing sequence may be represented by the following general formula:

**[Leu** and/or **Asp** and/or **Pro]-[Xaa-Lys-Xaa-Lys-$Y_{T/C}$]**

wherein each Xaa is independently selected from any amino acid, and $Y_{T/C}$ is an amino acid that is susceptible to cleavage by trypsin or chymotrypsin.

The invention also provides a method for preparing the peptide construct described above. In a specific embodiment, the construct is produced synthetically. In another embodiment, the construct is produced recombinantly.

In a further embodiment, the invention provides an isolated nucleic acid molecule encoding the peptide construct described above. The invention also provides an expression vector comprising such nucleic acid, as well as a host cell (*e.g.*, APC) and/or recombinant non-human host comprising such nucleic acid. According to a specific embodiment, the expression vector comprising the nucleic acid encoding the epitope construct of the invention may further comprise or may be combined with an APC targeting mechanism and may therefore be targeted directly to be expressed in the APCs of the host.

Further provided are pharmaceutical and vaccine compositions comprising an immunogenically effective amount of the peptide construct of the invention or a nucleic acid encoding such peptide construct and, optionally, further comprising a pharmaceutically acceptable adjuvant or excipient.

Also provided herein is a method for inducing or augmenting immunity (preferably, an antigen-specific cytotoxic T cell (CTL) immune response) induced by an antigen in a mammal comprising administering to the mammal the pharmaceutical or vaccine composition of the invention.

In a further embodiment, the invention provides a prophylactic and/or therapeutic method for treating a disease in a mammal comprising administering to the mammal at least one dose of the pharmaceutical or vaccine composition of the invention. As specified herein, this method can be useful for preventing and/or treating various neoplastic diseases, infections, autoimmune diseases, and the like. In a specific embodiment, the method of the invention is employed to treat cancer.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery that epitope sequences may be incorporated into a construct that (i) delivers the epitope(s) to antigen presenting cells (APCs), and (ii) facilitates appropriate processing of the epitopes once delivered to the APCs.

Specifically, the present invention provides a peptide construct comprising at least one epitope sequence, wherein the construct also includes or is associated with an antigen presenting cell (APC) targeting mechanism and is capable of stimulating an immune response such as, for example, a cytotoxic T-cell and/or helper T-cell and/or B-cell response.

The epitope contained in the peptide construct of the present invention can be, for example, a CD8+ T cell epitope, a CD4+ T cell epitope, a B cell epitope, or a combination thereof. As disclosed herein, the epitope can be derived from any antigen. For example, the epitope can be derived from a cancer antigen (also termed tumor-associated antigen or TAA), a viral antigen, a bacterial antigen, a protozoan antigen, or a fungal antigen. Preferably, the epitope is derived from a TAA. In one of the embodiments, the TAA is carcinoembryonic antigen (CEA).

The present invention also provides novel non-native epitopes designed for enhanced binding to MHC molecules and useful for modulating immune responses to the corresponding native peptides from which they are derived. Preferably, the synthetic antigenic peptide epitope sequences of the present invention differ from their native counterparts in that they contain alterations in amino acid sequence, relative to the native sequence, in the MHC class I binding domain, thereby conferring tighter binding to the MHC. Alternatively or in addition, they contain mutations in the putative T cell receptor (TCR)-binding domain, resulting in an increased affinity for the TCR. These differences from the native sequence confer advantages in the methods of the present invention over the native sequence, in that the synthetic antigenic peptide epitopes of the invention have enhanced immunomodulatory properties.

The constructs of the invention comprise at least one epitope sequence, or a combination of epitope sequences, which may be the same or different. Preferably, the

peptide construct of the invention is a polyepitope construct comprising more than one epitope, wherein said epitopes are derived from one or more different antigens. In polyepitope constructs of the invention, at least one epitope is preferably a cytotoxic T cell (CTL) epitope. In a specific embodiment, the construct represents an epitope string with epitopes arranged consecutively.

Also preferably, each of the epitopes in the peptide constructs of the invention is flanked on at least one side, and preferably on both sides, by a spacer (flanking) sequence comprising an internal processing sequence. The internal processing sequence is designed to facilitate the endosomal and/or lysosomal processing of the construct once it has reached the antigen presenting cell (APC).

Specific residues that may be included in the internal processing sequence include, but are not limited to, arginine (Arg, R), leucine (Leu, L), phenylalanine (Phe, F), aspartic acid (Asp, D), lysine (Lys, K), or proline (Pro, P). In a preferred embodiment, the internal processing sequence includes one or more Arg, Asp, and Pro residues. As endosomes and lysosomes possess proteolytic activity with substrate specificity similar to trypsin and chymotrypsin (see Hershko *et al.*, Ann. Rev. Biochem. 67: 425-79 (1998)), the epitope sequences included in the constructs of the present invention are preferably bounded by amino acid residues that act as substrates for those enzymes, such as lysine (Lys, K) or arginine (Arg, R) for trypsin, and phenylalanine (Phe, F), tryptophan (Trp, W), or tyrosine (Tyr, Y) for chymotrypsin.

In a specific embodiment, the internal processing sequence may further include a sequence signaling ubiquitination and/or an additional targeting signal(s) for transport to ER, endosomes or lysosomes. According to a specific embodiment, such additional targeting signal may contain one or more of the residues identified above for the internal processing sequence and is followed by an "internal sequence" which contains at least one lysine residue, and preferably two lysine residues at positions 3 and 17 of the internal sequence. Immediately following or adjacent to the internal sequence is the sequence of the epitope. One or more such additional targeting and ubiquitination sequences may be incorporated into the construct of the invention, the one or more targeting sequences being positioned in between each epitope sequence. With regard to the additional targeting sequences described above, reference is made to Bachmair *et al.*,

Science, 1986, 234: 179-186; Dantuma *et al.*, Nature Biotech., 2000, 18: 538-43; Velders *et al.*, J. Immunol., 2001, 166: 5366-5373; Toes *et al.*, Proc. Natl. Acad. Sci. USA, 1997, 94: 14660-14665; Thomson *et al.*, J. Virol., 1998, 72: 2246-2252; Ishioka *et al.*, J. Immunol., 1999, 162: 3915-3925; Thomson *et al.*, J. Immunol., 1998, 160: 1717-1723; and International Patent Publication Nos. WO 01/19408, WO 01/47541 and WO 97/35021.

According to the present invention, a "targeting mechanism" is a system that is capable of directing the constructs of the invention to APCs and facilitating proper internal processing of the constructs once inside the APCs. Specifically, the APC targeting mechanism is comprised of (i) an APC targeting sequence, which directs the construct to the APCs within the subject and, optionally (ii) a vehicle, such as, *e.g.*, a microsphere or liposome, that also acts as a targeting mechanism and/or preserves the viability of the construct until it has reached its intended APC and/or mediates the controlled release of the construct.

The APC targeting sequence may be covalently or non-covalently attached to the epitope. Preferably, the APC targeting sequence is covalently attached to the N-terminus or C-terminus of the sequence flanking respectively the first or the last epitope in the epitope string. In a specific embodiment, the APC targeting sequence is derived from a sequence capable of mediating interaction with such cell surface proteins as, for example, Heat Shock Protein Receptor (HSR) such as CD91, C-type lectin receptors such as Mannose Receptor (MR), DEC-205 and DC-SIGN, and IgG Fc receptor (FcR) such as Fc$\gamma$RI. In the Example 1 provided below, the APC targeting sequence is incorporated into the constructs at the C-terminus and comprises the gp96 sequence which interacts with the N-terminal p80 fragment of the alpha subunit of CD91, which is a receptor for heat shock proteins (see Binder *et al.*, Nature Immunol. 1: 151-55 (2000)).

Accordingly, as used herein, the term "construct" refers to a molecule consisting of at least one epitope sequence and at least one APC targeting sequence, which may be associated with the epitope sequence either covalently or non-covalently.

In a specific embodiment, the peptide construct of the invention may be represented by the following general formula:

**N-[APC targeting sequence]-[flanking sequence with internal processing sequence]-[epitope 1]-[flanking sequence with internal processing sequence]-[epitope 2]...[epitope n]-C**

or

**N-[epitope 1]-[flanking sequence with internal processing sequence]-[epitope 2]...[epitope n]-[flanking sequence with internal processing sequence]-[APC targeting sequence]-C**

wherein n is the number of epitopes in the construct and wherein the internal processing sequence may be represented by the following general formula:

**[Leu** and/or **Asp** and/or **Pro]-[Xaa-Lys-Xaa-Lys-$Y_{T/C}$]**

wherein each Xaa is independently selected from any amino acid, and $Y_{T/C}$ is an amino acid that is susceptible to cleavage by trypsin or chymotrypsin.

As described above, in addition to the APC targeting sequence, the targeting mechanism of the invention may include incorporating the constructs of the present invention into vehicles such as microspheres or liposomes that are internalized, *e.g.*, endocytosed, phagocytosed or pinocytosed, by the APCs, and then further processed by endosomes and/or lysosomes within the cells. In this regard, reference is made to U.S. Patent No. 6,312,731, which relates to a composition for inducing or potentiating an immune response, comprising an antigen and/or a bioactive agent encapsulated in a polymeric composition consisting of a polymer present in an amount sufficient to provide structural integrity to the composition, and a rapidly biodegradable component, a rapidly dissolving component, a rapidly swelling component, or a component that causes osmotic rupture of the encapsulated polymeric composition.

## Methods of Making Pepetide Constructs of the Invention

The invention also provides methods for preparing the peptide constructs described above. Such methods employ conventional techniques. Because the epitope-containing peptide constructs of the present invention will generally be short sequences, they can be routinely prepared by chemical synthesis using standard techniques. Particularly convenient are solid phase peptide synthesis techniques (See, *e.g.*, Steward and Young, eds. (1968) "Solid Phase Peptide Synthesis" Freemantle, San Francisco,

Calif.). Automated peptide synthesizers are commercially available (such as those manufactured by Perkin Elmer/Applied Biosystems, Inc., Model 430A or 431A, Foster City, Calif., USA.), as are the reagents required for their use. The peptides can also be prepared using recombinant techniques known to those of skill in the art using the host cells and nucleic acid vector systems described below.

Once an isolated peptide of the invention is obtained, it may be purified by standard methods including chromatography (*e.g.*, ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for protein purification. For immunoaffinity chromatography, an epitope may be isolated by binding it to an affinity column comprising antibodies that were raised against that peptide, or a related peptide of the invention, and were affixed to a stationary support. Alternatively, affinity tags such as hexa-His (Invitrogen), Maltose binding domain (New England Biolabs), influenza coat sequence (Kolodziej *et al.* (1991) Methods Enzymol. 194:508-509), and glutathione-S-transferase can be attached to the peptides of the invention to facilitate purification by passage over an appropriate affinity column. Isolated peptides can be physically characterized using such techniques as proteolysis, nuclear magnetic resonance, and x-ray crystallography.

Further provided are pharmaceutical and vaccine compositions comprising an immunogenically effective amount of the peptide construct of the invention or the nucleic acid encoding such polypeptide construct and, optionally, further comprising a pharmaceutically acceptable adjuvant or excipient.

Also provided herein is a method for inducing or augmenting immunity (preferably, an antigen-specific cytotoxic T cell (CTL) immune response) induced by an antigen in a mammal comprising administering to said mammal the pharmaceutical or vaccine composition of the invention.

In a further embodiment, the invention provides a prophylactic and/or therapeutic method for treating a disease in a mammal comprising administering to said mammal at least one dose of the pharmaceutical or vaccine composition of the invention. As specified herein, this method can be useful for preventing and/or treating various neoplastic diseases, infections, autoimmune diseases, and the like. In a specific embodiment, the method of the invention is employed to treat a cancer. The constructs of the present invention are preferably designed to elicit an anti-tumor immune response in an effort to decrease the rate of tumor growth, cause tumor regression, increase the time to relapse, and decrease mortality.

## General Definitions

The terms used in this specification generally have their ordinary meanings in the art within the context of this invention and in the specific context where each term is used. Certain terms are discussed below, or elsewhere in the specification, to provide additional guidance to the practitioner in describing the compositions and methods of the invention and how to make and use them.

The terms "cancer," "neoplasm," and "tumor" are used interchangeably and refer to cells that have undergone a malignant transformation that makes them pathological to the host organism. Primary cancer cells (that is, cells obtained from near the site of malignant transformation) can be readily distinguished from non-cancerous cells by well-established techniques, particularly histological examination. The definition of a "cancer cell", as used herein, includes not only a primary cancer cell, but any cell derived from a cancer cell ancestor. This includes metastasized cancer cells, and *in vitro* cultures and cell lines derived from cancer cells. When referring to a type of cancer that normally manifests as a solid tumor, a "clinically detectable" tumor is one that is detectable on the basis of tumor mass; *e.g.*, by such procedures as CAT scan, magnetic resonance imaging (MRI), X-ray, ultrasound, or palpation. Biochemical or immunologic findings alone may or may not be insufficient to meet this definition.

The term "vaccine" refers to a composition that can be used to elicit protective immunity in a recipient (*e.g.*, a composition comprising the peptide construct of the invention or the nucleic acid encoding such construct). It should be noted that to

be effective, a vaccine of the invention can elicit immunity in a portion of the population, as some individuals may fail to mount a robust or protective immune response, or, in some cases, any immune response. This inability may stem from the individual's genetic background or because of an immunodeficiency condition (either acquired or congenital) or immunosuppression (*e.g.*, treatment with immunosuppressive drugs to prevent organ rejection or suppress an autoimmune condition). Efficacy can be established in animal models.

The term "DNA vaccine" is an informal term of art, and is used herein to refer to a vaccine delivered by means of a recombinant vector. An alternative, and more descriptive term used herein is "vector vaccine" (since some potential vectors, such as retroviruses and lentiviruses are RNA viruses, and since in some instances non-viral RNA instead of DNA is delivered to cells through the vector). Generally, the vector is administered *in vivo*, but *ex vivo* transduction of appropriate antigen presenting cells, such as dendritic cells (DC), with administration of the transduced cells *in vivo*, is also contemplated.

The term "immunotherapy" refers to a treatment regimen based on activation of an antigen-specific immune response. A vaccine administration can be one form of immunotherapy.

"Immune response" broadly refers to the antigen-specific responses of lymphocytes. Any substance that can elicit an immune response is said to be "immunogenic" and is referred to as an "immunogen". An immune response of this invention can be humoral (via antibody activity) or cell-mediated (via T cell activation) or both.

As used herein, the term "immunogenic" means that the agent (*e.g.*, protein, peptide, polysaccharide, glycoprotein, glycolipid, nucleic acid, or combination thereof) is capable of eliciting a humoral or cellular immune response, and preferably both, when administered to an animal having an immune system. An immunogenic peptide is also antigenic. A molecule is "antigenic" when it is capable of specifically interacting with an antigen recognition molecule of the immune system, such as an immunoglobulin (antibody) or T cell receptor (TCR). Antigenic ligand sequences that specifically interact with antibodies, MHC class I, and MHC class II molecules are

described herein. An antigenic portion of a peptide, also called herein an epitope, can be that portion that is immunodominant for antibody or T cell receptor recognition, or it can be a portion used to generate an antibody to the molecule by conjugating the antigenic portion to a carrier polypeptide for immunization. A molecule that is antigenic need not itself be immunogenic, *i.e.*, capable of eliciting an immune response, without a carrier, adjuvant or excipient.

## Antigens and Epitopes of the Invention

The term "antigen" refers to any agent (*e.g.*, protein, peptide, polysaccharide, glycoprotein, glycolipid, nucleic acid, or combination thereof) that, when introduced into a host having an immune system (directly or upon expression as in, *e.g.*, DNA vaccines), is recognized by the immune system of the host and is capable of eliciting a specific immune reaction.

The terms "tumor-associated antigen (TAA)" and "tumor-specific antigen (TSA)" are used interchangeably and refer to an antigenic peptide that is associated with a tumor. As described in the Background Section, TAAs include, for example, mutated cellular proteins such as mutated tumor suppressor gene products, oncogene products (including fusion proteins), and foreign proteins such as viral gene products. Non-mutated cellular proteins may also be TAAs if they are expressed aberrantly (*e.g.*, in an inappropriate subcellular compartment) or in supernormal quantities.

The term "epitope" or "antigenic determinant" refers to any portion of an antigen recognized either by B cells, or T cells, or both. Preferably, interaction of an epitope with an antigen recognition site of an immunoglobulin or TCR involves antigen-specific immune recognition.

CTL epitope sequences, T helper cell sequences, and B cell epitope sequences may be included in the constructs of the invention. The epitopes used in immunogenic (*e.g.*, vaccine) compositions of the instant invention can be derived from any antigen present in a eukaryotic cell (*e.g.*, tumor, parasite, fungus), bacterial cell, viral particle, or any portion thereof.

Examples of preferred antigens of the present invention include tumor-associated antigens (TAAs) such as ErbB receptors, Melan A [MART1], gp100,

tyrosinase, TRP-1/gp 75, and TRP-2 (in melanoma; for additional examples, *see* also a list of antigens provided in Storkus and Zarour, Forum (Genova), 2000 Jul-Sep, 10(3):256-270); MAGE-1 and MAGE-3 (in bladder, head and neck, and non-small cell carcinoma); HPV EG and E7 proteins (in cervical cancer); Mucin [MUC-1] (in breast, pancreas, colon, and prostate cancers); prostate-specific antigen [PSA] (in prostate cancer); carcinoembryonic antigen [CEA] (in colon, breast, lung, thyroid, and gastrointestinal cancers), P1A tumor antigen (*e.g.*, as disclosed in International Patent Publication No. WO 98/56919), and such shared tumor-specific antigens as MAGE-2, MAGE-4, MAGE-6, MAGE-10, MAGE-12, BAGE-1, CAGE-1,2,8, CAGE-3 to 7, LAGE-1, NY-ESO-1/LAGE-2, NA-88, and GnTV (*see, e.g.*, International Patent Publication No. WO 98/56919).

In a specific embodiment, the constructs of the present invention include at least one epitope derived from carcinoembryonic antigen (CEA). CEA is associated with neoplasms of epithelial origin, including carcinomas of the gastrointestinal tract, breast, lung, and thyroid, and therefore, constructs of the invention that include CEA epitopes may be used to threat neoplasms of epithelial origin.

Other antigens of the invention include but are not limited to (i) protozoan antigens such as those derived from *Plasmodium* sp., *Toxoplasma* sp., *Pneumocystis carinii*, *Leishmania* sp., and *Trypanosoma* sp.; (ii) viral protein or peptide antigens such as those derived from influenza virus (*e.g.*, surface glycoproteins hemagluttinin (HA) and neuraminidase (NA) or the nucleoprotein (NP) as described in Bodmer *et al.*, Cell, 52:253, 1988 and Tsuji *et al.*, J. Virol. 72: 6907-6910, 1998 or NP CTL epitopes as described in Gould *et al.*, J. Virol., 65:5401, 1991; Murata *et al.*, Cell Immunol., 173:96-107, 1996, and PCT Application No. WO 98/56919); immunodeficiency virus (*e.g.*, a simian immunodeficiency virus (SIV) antigen [*e.g.*, SIV-env CTL epitope as disclosed in PCT Application No. WO 98/56919], or a human immunodeficiency virus antigen (HIV-1) such as gp120 CTL epitopes as disclosed, *e.g.*, in PCT Application No. WO 98/56919], gp160, p18 antigen [*e.g.*, CD8+ T cell epitopes and gp41 CTL epitopes as disclosed, *e.g.*, in PCT Application No. WO 98/56919], Gag p24 CD8+ T cell epitopes, Gag p17 CD8+ T cell epitopes, Tat, Pol, Nef CTL epitopes as disclosed, *e.g.*, in PCT Application No. WO 98/56919], and Env CTL epitopes as disclosed, *e.g.*, in PCT

Application No. WO 98/56919]; herpesvirus (*e.g.*, a glycoprotein, for instance, from feline herpesvirus, equine herpesvirus, bovine herpesvirus, pseudorabies virus, canine herpesvirus, herpes simplex virus (HSV, *e.g.*, HSV tk, gB, gD), herpes zoster virus, Marek's Disease Virus, herpesvirus of turkeys (HVT), cytomegalovirus (CMV), or Epstein-Barr virus); hepatitis C virus; human papilloma virus (HPV); human T cell leukemia virus (HTLV-1); bovine leukemia virus (*e.g.*, gp51,30 envelope antigen); feline leukemia virus (FeLV) (*e.g.*, FeLV envelope protein, a Newcastle Disease Virus (NDV) antigen, *e.g.*, HN or F); rous associated virus (such as RAV-1 env); infectious bronchitis virus (*e.g.*, matrix and/or preplomer); flavivirus (*e.g.*, a Japanese encephalitis virus (JEV) antigen, a Yellow Fever antigen, or a Dengue virus antigen); Morbillivirus (*e.g.*, a canine distemper virus antigen, a measles antigen, or rinderpest antigen such as HA or F); rabies (*e.g.*, rabies glycoprotein G); parvovirus (*e.g.*, a canine parvovirus antigen); hepatitis C virus (HCV); poxvirus (*e.g.*, an ectromelia antigen, a canary poxvirus antigen, or a fowl poxvirus antigen such as chicken pox virus varicella zoster antigen); infectious bursal disease virus (*e.g.*, VP2, VP3, or VP4); Hantaan virus; mumps virus, and measles virus; (iii) bacterial antigens such as *Mycobacterium tuberculosis*-specific (*e.g.*, Bacillus Calmette-Guérin [BCG] - 38kD protein; antigen 85 complex [as described in Klein *et al.*, J. Infect. Dis., 183:928-34, 2001], *see* also a list of antigens in Klein and McAdam, Arch. Immunol. Ther. Exp. (Warsz.), 47:313-320, 1999), *Listeria monocytogenes*-specific (*e.g.*, as disclosed in Finelli *et al.*, Immunol. Res., 19:211-223, 1999), *Salmonella typhii*-specific, *Shigella flexineri*-specific, staphylococcus-specific, streptococcus-specific, pneumococcus-specific (*e.g.*, PspA [*see* PCT Publication No. WO 92/14488]), *Neisseria gonorrhea*-specific, *Borrelia*-specific (*e.g.*, OspA, OspB, OspC antigens of *Borrelia* associated with Lyme disease such as *Borrelia burgdorferi*, *Borrelia afzelli*, and *Borrelia garinii* [*see, e.g.*, U.S. Patent No. 5,523,089; PCT Application Nos. WO 90/04411, WO 91/09870, WO 93/04175, WO 96/06165, WO93/08306; PCT/US92/08697; Bergstrom *et al.*, Mol. Microbiol., 3: 479-486, 1989; Johnson *et al.*, Infect. and Immun. 60: 1845-1853, 1992; Johnson *et al.*, Vaccine 13: 1086-1094, 1995; *The Sixth International Conference on Lyme Borreliosis: Progress on the Development of Lyme Disease Vaccine*, Vaccine 13: 133-135, 1995]), *A. pertussis*-specific, *S. parathyphoid* A and B-specific, *C. diphtheriae*-specific, *C. tetanus*-specific, *C. botulinum*-specific, *C. perifringens*-specific,

*A. anthracis*-specific, *A. pestis*-specific, *V. cholera*-specific, *H. influenzae*-specific, *T. palladium*-specific, *Chlamydia trachomatis*-specific (*e.g.*, as disclosed in Kim *et al.*, J. Immunol., 162:6855-6866, 1999), and pseudomonas-specific proteins or peptides; and (iv) fungal antigens such as those isolated from candida (*e.g.*, 65kDa mannoprotein [MP65] from *Candida albicans*), trichophyton, or ptyrosporum.

The foregoing list of antigens is intended as exemplary, as the antigen of interest can be derived from any animal or human pathogen or tumor. With respect to DNA encoding pathogen-derived antigens of interest, attention is directed to, *e.g.*, U.S. Patent Nos. 4,722,848; 5,174,993; 5,338,683; 5,494,807; 5,503,834; 5,505,941; 5,514,375; 5,529,780; U.K. Patent No. GB 2 269 820 B; and PCT Publication Nos. WO 92/22641; WO 93/03145; WO 94/16716; WO 96/3941; PCT/US94/06652. With respect to antigens derived from tumor viruses, reference is also made to *Molecular Biology of Tumor Viruses, RNA Tumor Viruses*, Second Edition, Edited by Weiss *et al.*, Cold Spring Harbor Laboratory Press, 1982. For a list of additional antigens useful in the compositions of the invention *see* also Stedman's Medical Dictionary (24th edition, 1982).

To provide additional antigen-derived B and T cell epitopes for use in the constructs of the present invention, these epitopes can be identified by one or a combination of several methods well known in the art, such as, for example, by (i) fragmenting the antigen of interest into overlapping peptides using proteolytic enzymes, followed by testing the ability of individual peptides to bind to an antibody elicited by the full-length antigen or to induce T cell or B cell activation (*see, e.g.*, Janis Kuby, Immunology, pp. 79-80, W. H. Freeman, 1992); (ii) preparing synthetic peptides, the sequences of which are segments or analogs of a given antigen (*see, e.g.*, Alexander *et al.*, Immunity, 1: 751-61, 1994; Hammer *et al.*, J. Exp. Med., 180: 2353-8, 1994), or constructs based on such segments, or analogs linked or fused to a carrier or a heterologous antigen and testing the ability of such synthetic peptides to elicit antigen-specific antibodies or T cell activation (*e.g.*, testing their ability to interact with MHC class II molecules both *in vitro* and *in vivo* [*see, e.g.*, O'Sullivan *et al.*, J. Immunol., 147: 2663-9, 1991; Hill *et al.*, J. Immunol., 147: 189-197, 1991]); for determination of T cell epitopes, peptides should be at least 8 to 10 amino acids long to occupy the groove of the

MHC class I molecule and at least 13 to 25 amino acids long to occupy the groove of MHC class II molecule, preferably, the peptides should be longer; these peptides should also contain an appropriate anchor motif which will enable them to bind to various class I or class II MHC molecules with high enough affinity and specificity to generate an immune response (*see* Bocchia *et al.*, Blood, 85: 2680-2684, 1995; Englehard, Ann. Rev. Immunol., 12: 181, 1994); (iii) sequencing peptides associated with purified MHC molecules (*see, e.g.*, Nelson *et al.*, Proc. Natl. Acad. Sci. USA, 94:628-33, 1997); (iv) screening a peptide display library for high-affinity binding to MHC class II molecules, TCR, antibodies raised against a full-length antigen, etc. (*see, e.g.*, Hammer *et al.*, J. Exp. Med., 176:1007-13, 1992); (v) computationally analyzing different protein sequences to identify, *e.g.*, hydrophilic stretches (hydrophilic amino acid residues are often located on the surface of the protein and are therefore accessible to the antibodies) and/or high-affinity TCR or MHC class II allele-specific motifs, *e.g.*, by comparing the sequence of the protein of interest with published structures of peptides associated with the MHC molecules (Mallios, Bioinformatics, 15:432-439, 1999; Milik *et al.*, Nat. Biotechnol., 16: 753-756, 1998; Brusic *et al.*, Nuc. Acids Res, 26: 368-371, 1998; Feller and de la Cruz, Nature, 349: 720-721, 1991); (vi) performing an x-ray crystallographic analysis of the native antigen-antibody complex (Janis Kuby, Immunology, p. 80, W. H. Freeman, 1992), and (vii) generating monoclonal antibodies to various portions of the antigen of interest, and then ascertaining whether those antibodies attenuate *in vitro* or *in vivo* growth of the pathogen or tumor from which the antigen was derived (*see* U.S. Patent No. 5,019,384 and references cited therein).

## Non-Native Epitopes and Methods for Their Generation

A "native" or "wild-type" or "natural" or "self" epitope is an epitope, which has been isolated from a natural biological source, and which can be recognized by the immune system.

An "altered" or "non-natural" or "non-native" or "modified" or "synthetic" epitope is one having a primary sequence that is different from that of the corresponding native epitope. Although using epitopes with non-native peptide sequences may induce immune responses that are not specific for the intended target,

according to the present invention, the use of non-native epitopes is preferred for the following reasons. First, it is likely that native peptide sequences derived from antigens (*e.g.*, tumor-associated antigens (TAAs) or self-antigens in autoimmune disorders) may be recognized by the immune system as self and patients may be tolerized to these peptide sequences. Second, many native peptide sequences do not bind with high affinity to the MHC molecules. As it has been shown that the strength of the immune response to a particular epitope is related to the binding affinity of the epitope to the MHC class I complex (*i.e.*, stronger immune responses can be induced by peptides that bind with higher affinity to the MHC molecules), it is likely that the immune response to native epitopes will be lower than the immune responses to non-native epitopes that bind with higher affinity to the MHC molecules.

Non-native peptide epitopes can be produced using any method known in the art. The following provides non-limiting examples of such methods. In addition, modifications or combinations of any of the following methods can be used.

The non-native epitopes may be derived from native epitopes (*e.g.*, using site-directed mutagenesis) by modifying them in any way known in the art, as long as the modification does not completely prevent their ability to generate an immune response. In particular, the constructs of the invention may have one or more amino acid substitutions, deletions, or insertions. For example, such amino acid substitutions may include substitutions of functionally equivalent amino acid residues. One or more amino acid residues can be substituted by another amino acid of a similar polarity that acts as a functional equivalent resulting in a silent alteration. Substitutes for an amino acid may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

Additionally, one or more amino acid residues can be substituted by a nonclassical amino acid or chemical amino acid analogs. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, alpha-amino

isobutyric acid, 4-aminobutyric acid, 2-aminobutyric acid, 6-amino hexanoic acid, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, beta-alanine, fluoro amino acids, designer amino acids such as beta methyl amino acids, C-α-methyl amino acids, N-α-methyl amino acids, and amino acid analogs in general. Peptides of the invention may also comprise various "designer" amino acids (*e.g.*, β-methyl amino acids, C-α-methyl amino acids, and N-α-methyl amino acids, etc.) to convey special properties to peptides. Additionally, by assigning specific amino acids at specific coupling steps, peptides with α-helices, β-turns, β-sheets, γ-turns, and cyclic peptides can be generated. Generally, it is believed that α-helical secondary structure or random secondary structure is preferred.

The following non-classical amino acids may be incorporated in the peptides of the invention in order to introduce particular conformational motifs: 1,2,3,4-tetrahydroisoquinoline-3-carboxylate (Kazmierski *et al.* (1991) J. Am. Chem. Soc. 113:227S-2283); (2S,3S)-methyl-phenylalanine, (2S,3R)-methyl-phenylalanine, (2R,3S)-methyl-phenylalanine and (2R,3R)-methyl-phenylalanine; 2-aminotetrahydro-naphthalene-2-carboxylic acid (Landis (1989) Ph.D. Thesis, University of Arizona); hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylate (Miyake *et al.* (1989) J. Takeda Res. Labs 43:53-76), histidine isoquinoline carboxylic acid (Zechel *et al.* (1991) Int. J. Pep. Protein Res. 43), and HIC (histidine cyclic urea) (Dharanipragada).

The following amino acid analogs and peptidomimetics may be incorporated into a peptide to induce or favor specific secondary structures: LL-Acp (LL-3-amino-2-propenidone-6-carboxylic acid), a β-turn inducing dipeptide analog (Kemp *et al.* (1985) J. Org. Chem. 50:5834-5838); β-sheet inducing analogs (Kemp *et al.* (1988) Tetrahedron Lett. 29:5081-5082); β-turn inducing analogs (Kemp *et al.* (1988) Tetrahedron Lett. 29:5057-5060); α-helix inducing analogs (Kemp *et al.* (1988) Tetrahedron Lett. 29:4935-4938); γ-turn inducing analogs (Kemp *et al.* (1989) J. Org. Chem. 54:109:115); and analogs provided by the following references: Nagai and Sato (1985) Tetrahedron Lett. 26:647-650; DiMaio *et al.* (1989) J. Chem. Soc. Perkin Trans. p. 1687; also a Gly-Ala turn analog (Kahn *et al.* (1989) Tetrahedron Lett. 30:2317); amide bond isostere (clones *et al.* (1988) Tetrahedron Lett. 29:38S3-38S6); tretrazol (Zabrocki

*et al.* (1988) J. Am. Chem. Soc. 110:587S-5880); DTC (Samanen *et al.* (1990) Int. J. Protein Pep. Res. 35:501:509); and analogs taught in Olson *et al.* (1990) J. Am. Chem. Sci. 112:323-333 and Garvey *et al.* (1990) J. Org. Chem. 56:436. Conformationally restricted mimetics of beta turns and beta bulges, and peptides containing them, are described in U.S. Patent No. 5,440,013.

In addition, the present invention envisions preparing peptides that have more well-defined structural properties, and the use of peptidomimetics, and peptidomimetic bonds, such as ester bonds, to prepare peptides with novel properties. In another embodiment, a peptide may be generated that incorporates a reduced peptide bond, *i.e.*, R1--CH2 NH--R2, where R1, and R2 are amino acid residues or sequences. A reduced peptide bond may be introduced as a dipeptide subunit. Such a molecule would be resistant to peptide bond hydrolysis, *e.g.*, protease activity. Such molecules would provide ligands with unique function and activity, such as extended half-lives *in vivo* due to resistance to metabolic breakdown, or protease activity.

In a specific embodiment, non-native epitopes are derived from native epitopes recombinantly by introducing post-translational modifications *e.g.*, by phosphorylation, glycosylation, cross-linking, acylation, proteolytic cleavage, linkage to an antibody molecule, membrane molecule or other epitope (Ferguson *et al.* Ann. Rev. Biochem. 1988, 57:285-320).

For generation of large numbers of new non-native epitope sequences, bacteriophage "phage display" libraries ($10^6$-$10^8$ chemical entities) can be constructed (Scott and Smith (1990) Science 249:386-390; Cwirla *et al.* (1990) Proc. Natl. Acad. Sci. USA 87:6378-6382; Devlin *et al.* (1990) Science 249:404-406). Other methods involve primarily chemical synthesis methods, of which the Geysen method (Geysen *et al.* (1986) Molecular Immunology 23:709-715; Geysen *et al.* (1987) J. Immunologic Method 102:259-274) and the method of Fodor *et al.* ((1991) Science 251:767-773) are examples. See also Furka *et al.* (1988) 14th International Congress of Biochemistry, Volume 5. Abstract FR:013; Furka, (1991) Int. J. Peptide Protein Res. 37:487-493). See also U.S. Pat. Nos. 4,631,211 and 5,101,175, which describe methods to produce a mixture of peptides. Other methods which can be employed involve use of synthetic libraries (Needels *et al.* (1993) Proc. Natl. Acad. Sci. USA 90:10700-4; Ohlmeyer *et al.* (1993)

Proc. Natl. Acad. Sci. USA 90:10922-10926; International Patent Publication No. WO 92/00252) and techniques based on cDNA subtraction or differential display (Hedrick *et al.* (1984) Nature 308:149; Lian and Pardee (1992) Science 257:967). Another technique which can be used is the "pepscan" technique (Van der Zee (1989) Eur. J. Immunol. 19:43-47), in which several dozens of peptides are simultaneously synthesized on polyethylene rods arrayed in a 96-well microtiter plate pattern, similar to an indexed library in that the position of each pin defines the synthesis history on it. Peptides are then chemically cleaved from the solid support and supplied to irradiated syngeneic thymocytes for antigen presentation. A cloned CTL line can then be tested for reactivity in a proliferation assay monitored by $^3$H-thymidine incorporation.

In a specific embodiment non-natural epitopes of the present invention are generated from a combinatorial library of oligopeptides attached to solid phase supports using SPHERE™ technology, which is described in the International Patent Publication No. WO 97/35035, U.S. Patent Nos. 6,528,060 and 6,338,945, and Published U.S. Application No. 2002/0164346. This approach utilizes combinatorial peptide libraries synthesized on polystyrene beads wherein each bead contains a pure population of a unique peptide that can be chemically released from the beads in discrete aliquots. The peptides attached to a single bead have essentially the same amino acid sequence. The synthesis history of each peptide bead may be recorded on each solid support in a code of inert molecular tags, such that beads of interest can be rapidly and efficiently decoded. A photocleavable crosslinker allows release of some of the oligopeptide by exposure to UV light. Molecular tags, if present, remain covalently bound to the beads for post-assay analysis. Released peptide from pooled bead arrays are screened using methods to detect T cell activation, including, for example, $^3$H-thymidine incorporation (for CD4+ or CD8+ T cells), $^{51}$Cr-release assay (for CTLs), or IL-2 production (for CD4+ T cells) to identify peptide pools capable of activating a T cell of interest. By utilizing an iterative peptide pool/releasing strategy, it is possible to screen more than $10^7$ peptides in just a few days. Analysis of residual peptide on the corresponding positive beads (>100 pmoles) allows rapid and unambiguous identification of the epitope sequence.

As specified above, non-native epitopes of the invention may bind to

MHC molecules with higher affinity than the corresponding native epitopes. Binding of the epitopes of the invention to MHC molecules can be measured by methods that are known in the art and include, but are not limited to, calculating the affinity based on an algorithm (see, for example, Parker *et al.* (1992) J. Immunol. 149:3580-3587); and experimentally determining binding affinity (see, *e.g.*, Tan *et al.* (1997) J. Immunol. Meth. 209:25-36). For example, the relative binding of a peptide to an MHC molecule can be measured on the basis of binding of a radiolabeled standard peptide to detergent-solubilized MHC molecules, using various concentrations of test peptides (*e.g.*, ranging from 100 mM to 1 nM). For example, MHC class I heavy chain and $\beta$2-microglobulin may be co-incubated with a fixed concentration (*e.g.*, 5 nM) of radiolabeled native (control) peptide and various concentrations of a corresponding non-native peptide for a suitable period of time (*e.g.*, 2 hours to 72 hours) at room temperature in the presence of a mixture of protease inhibitors. The percent MHC-bound radioactivity is determined by gel filtration. The $IC_{50}$ (concentration of test peptide which results in 50% inhibition of binding of control peptide) is calculated for each peptide.

As specified above, non-native epitopes of the invention may also bind to TCRs with higher affinity than the corresponding native epitopes. Methods for determining binding affinity to TCRs are known in the art and include, but are not limited to, those described in al-Ramadi *et al.* (1992) J. Imunol. 155(2):662-673; and Zuegel *et al.* (1998) J. Immunol. 161(4):1705-1709.

## Immune Effector Cells

The term "immune effector cells" refers to cells capable of binding an antigen and which thereby mediate an immune response. These cells include, but are not limited to, T cells, B cells, monocytes, macrophages, NK cells, and cytotoxic T lymphocytes (CTLs). The activation of T cells by professional antigen presenting cells (APCs) leads to their proliferation and the differentiation of their progeny into armed effector T cells which can act on any target cell that displays antigen on its surface. Effector T cells can mediate a variety of functions. One set of important functions is the killing of cells, *e.g.*, cancer cells or cells infected by viruses or bacteria, by CD8+ CTLs, and the activation of macrophages by $T_H1$ cells, which together make up cell-mediated

immunity. In addition, B cells are activated by $T_H2$ cells to produce different types of antibody, thus driving the humoral immune response.

The term “antigen presenting cells” or “APCs” refers to a class of cells capable of presenting one or more antigens in the form of antigen-MHC complexes recognizable by specific effector cells of the immune system, and thereby inducing an effective cellular immune response against the antigen or antigens being presented. While many types of cells may be capable of presenting antigens on their cell surface for T-cell recognization, only professional APCs have the capacity to present antigens in an efficient amount and further to activate T-cells for CTL responses. APCs include, for example, macrophages, B-cells and dendritic cells (DCs).

The term “dendritic cells” or “DCs” refers to a diverse population of morphologically similar cell types found in a variety of lymphoid and non-lymphoid tissues (Steinman (1991) Ann. Rev. Immunol. 9:271-296). DCs constitute the most potent and preferred APCs in the organism. A subset, if not all, of DCs are derived from bone marrow progenitor cells, circulate in small numbers in the peripheral blood and appear either as immature Langerhans' cells or terminally differentiated mature cells. DCs are professional APCs that efficiently capture antigens in the peripheral tissues and process these antigens to form MHC-peptide complexes. After antigen uptake, these immature DCs acquire the unique capacity to migrate from the periphery to the T cell areas of secondary lymphoid organs. As the cells travel, they mature and alter their profile of cell surface molecules, to attract resting T cells and present their antigenic load (Shaw *et al.*, (1986) Nature 323, 262-264; Adema *et al.*, (1997) Nature 387, 713-717; Banchereau and Steinman, (1998) Nature 392, 245-252). While the DCs can be differentiated from monocytes, they possess distinct phenotypes. For example, a particular differentiating marker, CD14 antigen, is not found in DCs but is possessed by monocytes. Also, mature DCs are not phagocytic, whereas the monocytes are strongly phagocytosing cells. It has been shown that DCs provide all the signals necessary for T cell activation and proliferation.

T cells recognize proteins only when they have been cleaved into smaller peptides and are presented in a complex called the "major histocompatability complex (MHC)" located on the another cell's surface. The terms “major histocompatability

complex" or "MHC" refers to a complex of genes encoding cell-surface molecules that are required for antigen presentation to T cells and for rapid graft rejection. In humans, the MHC is also known as the "human leukocyte antigen" or "HLA" complex. The proteins encoded by the MHC are known as "MHC molecules" and are classified into class I and class II MHC molecules. Class I MHC includes membrane heterodimeric proteins made up of an $\alpha$ chain encoded in the MHC noncovalently linked with the $\beta$2-microglobulin. Class I MHC molecules are expressed by nearly all nucleated cells and have been shown to function in antigen presentation to CD8+ T cells, *i.e.*, T cells which have the CD8 protein on their surface. CD8+ T cells, bind specifically to the MHC class I/peptide complexes via the T cell receptor (TCR). This leads to cytolytic effector activities. Class I molecules include HLA-A, B and C in humans. Class II MHC molecules also include membrane heterodimeric proteins consisting of non-covalently associated $\alpha$ and $\beta$ chains. Class II MHC complexes are found only on APCs and are used to present peptides from antigens which have been endocytosed by APCs. T cells which have the CD4 protein on their surface, *i.e.*, CD4+ T cells, bind to the MHC class II/peptide complexes via TCR. This leads to the synthesis of specific cytokines which stimulate an immune response. Class II MHCs in humans include HLA-DP, DQ, and DR. Those of skill in the art are familiar with the serotypes and genotypes of the HLA (see Rammensee, H.G., Bachmann, J., and Stevanovic, S. MHC Ligands and Peptid Motifs (1977) Chapman & Hall Publishers; Schreuder *et al.*, The HLA dictionary, Tissue Antigens 1999, 54:409-437). To be effectively recognized by the immune system via MHC class I presentation, an antigenic polypeptide has to contain an epitope of at least about 8 to 10 amino acids, while to be effectively recognized by the immune system via MHC class II presentation, an antigenic polypeptide has to contain an epitope of at least about 13 to 25 amino acids. *See, e.g., Fundamental Immunology*, 3rd Edition, W.E. Paul ed., 1999, Lippincott-Raven Publ.

The term "native antibodies" or "immunoglobulins" refers to usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. In most classes of immunoglobulin molecules, each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different

immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (VH) followed by a number of constant domains. Each light chain has a variable domain (VL) at one end and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains (Clothia *et al.*, J Mol. Biol., 186: 651-663, 1985; Novotny and Haber, Proc. Natl. Acad. Sci. USA, 82: 4592-4596, 1985).

The term "antibody" or "Ab" is used in the broadest sense and specifically covers not only native antibodies but also single monoclonal antibodies (including agonist and antagonist antibodies), antibody compositions with polyepitopic specificity, as well as antibody fragments (*e.g.*, Fab, $F(ab')_2$, scFv and Fv), so long as they exhibit the desired biological activity.

The term "cross-reactive" is used to describe compounds of the invention which are functionally overlapping. More particularly, the immunogenic properties of a native epitope and/or immune effector cells activated thereby are shared to a certain extent by the non-native epitope derived therefrom such that the non-native epitope is "cross-reactive" with the native epitope and/or the immune effector cells activated thereby. For purposes of this invention, cross-reactivity may be manifested at multiple levels: (i) at the epitope level, *e.g.*, the non-native epitopes can bind the TCR of and activate native epitope-specific CTLs; (ii) at the T cell level, *i.e.*, non-native epitopes of the invention bind the TCR and activate a population of T cells (distinct from the population of native epitope-specific CTLs) which can effectively target and lyse cells displaying the native epitope; and (iii) at the antibody level, *e.g.*, "anti"-non-native epitope antibodies can detect, recognize and bind the native epitope and initiate effector mechanisms in an immune response.

**Therapeutic Definitions**

As used herein, the term "inducing an immune response in a subject" is a term well understood in the art and means that an increase in an immune response to an

antigen (or epitope) can be detected or measured, after introducing the construct into the subject, relative to the immune response (if any) before introduction of the construct into the subject. Methods of determining whether an immune response to a given antigen (or epitope) has been induced are well known in the art. For example, antigen-specific antibody can be detected using any of a variety of immunoassays known in the art, including, but not limited to, ELISA, wherein, for example, binding of an antibody in a sample to an immobilized antigen (or epitope) is detected with a detectably labeled second antibody (*e.g.*, enzyme-labeled mouse anti-human Ig antibody).

The term "treat" is used herein to mean to relieve or alleviate at least one symptom of a disease or condition in a subject. Within the meaning of the present invention, the term "treat" may mean to prolong the prepatency, *i.e.*, the period between infection or neoplastic transformation and clinical manifestation of a disease. The term "protect" is used herein to mean prevent or treat, or both, as appropriate, the development or continuance of a disease in a subject. Within the meaning of the present invention, the disease is selected from the group consisting of malignancy (*e.g.*, solid or blood tumors such as sarcomas, carcinomas, gliomas, blastomas, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, lymphoma, leukemia, melanoma, etc.), infection (*e.g.*, viral, bacterial, parasitic, or fungal) and an autoimmune disease (*e.g.*, most forms of arthritis, ulcerative colitis, asthma, or multiple sclerosis). For example, if the epitope(s) incorporated into the construct of the invention is(are) derived from CEA (carcinoembryonic antigen), which is associated with neoplasms of epithelial origin, including carcinomas of the gastrointestinal tract, breast, lung, and thyroid, the construct of the invention may be administered to prevent or treat neoplasms of epithelial origin in a subject in need of such treatment. Therefore, the intended use of the constructs of the invention will largely depend on the origin of the epitope(s). Prophylactic administration of the vaccine can protect the recipient subject at risk of developing such a cancer, *e.g.*, as determined from family history.

The phrase "pharmaceutically acceptable", as used in connection with compositions of the invention, refers to molecular entities and other ingredients of such compositions that are physiologically tolerable and do not typically produce untoward reactions when administered to a human. Preferably, as used herein, the term

"pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in mammals, and more particularly in humans.

The terms "adjuvant" and "immunoadjuvant" are used interchangeably in the present invention and refer to a compound or mixture that may be non-immunogenic when administered to a host alone, but that augments the host's immune response to another antigen when administered conjointly with that antigen.

The adjuvant of the invention can be administered as part of a pharmaceutical or vaccine composition comprising an antigen or as a separate formulation. The adjuvants of the invention include, but are not limited to, oil-emulsion and emulsifier-based adjuvants such as complete Freund's adjuvant, incomplete Freund's adjuvant, MF59, or SAF; mineral gels such as aluminum hydroxide (alum), aluminum phosphate or calcium phosphate; microbially-derived adjuvants such as cholera toxin (CT), pertussis toxin, *Escherichia coli* heat-labile toxin (LT), mutant toxins (*e.g.*, LTK63 or LTR72), *Bacille Calmette-Guerin* (BCG), *Corynebacterium parvum*, DNA CpG motifs, muramyl dipeptide, or monophosphoryl lipid A; particulate adjuvants such as immunostimulatory complexes (ISCOMs), liposomes, biodegradable microspheres, or saponins (*e.g.*, QS-21); cytokines such as IFN-γ, IL-2, IL-12 or GM-CSF; synthetic adjuvants such as nonionic block copolymers, muramyl peptide analogues (*e.g.*, N-acetyl-muramyl-L-threonyl-D-isoglutamine [thr-MDP], N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine, N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-[1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy]-ethylamine), polyphosphazenes, or synthetic polynucleotides, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, hydrocarbon emulsions, or keyhole limpet hemocyanins (KLH). Preferably, these adjuvants are pharmaceutically acceptable for use in humans.

Within the meaning of the present invention, the term "conjoint administration" is used to refer to administration of an immune adjuvant and an antigen simultaneously in one composition, or simultaneously in different compositions, or sequentially.

The term "excipient" applied to pharmaceutical or vaccine compositions of the invention refers to a diluent or vehicle with which an antigen-containing compound

and/or an adjuvant is administered. Such pharmaceutical excipients can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution, saline solutions, and aqueous dextrose and glycerol solutions are preferably employed as excipients, particularly for injectable solutions. Suitable pharmaceutical excipients are described in "Remington's Pharmaceutical Sciences" by E.W. Martin, $18^{th}$ Edition.

The term "protective immunity" refers to an immune response in a host animal (either active/acquired or passive/innate, or both) which leads to inactivation and/or reduction in the load of an antigen and to generation of immunity (that is acquired, *e.g.*, through production of antibodies), which prevents or delays the development of a disease upon repeated exposure to the same or a related antigen. A "protective immune response" involves humoral (antibody) immunity or cellular immunity, or both, effective, *e.g.*, to reduce a tumor burden in an immunized (vaccinated) subject or to eliminate or reduce, or slow the increase in, the load of a pathogen or infected cell (or produce any other measurable alleviation of the infection). Within the meaning of the present invention, protective immunity may be partial. In specific embodiments of the invention, protective immunity is reflected by any improvement in any condition or symptom being treated, including any one of the following: a slowing of disease progression, increasing length to relapse, decreased rate of tumor growth, tumor regression, decreased mortality, etc.

As used herein, the term "augment the immune response" means enhancing or extending the duration of the immune response, or both.

The phrase "enhance immune response" within the meaning of the present invention refers to the property or process of increasing the scale and/or efficiency of immunoreactivity to a given antigen, said immunoreactivity being either humoral or cellular immunity, or both. An immune response is believed to be enhanced, if any measurable parameter of antigen-specific immunoreactivity (*e.g.*, antibody titer, T cell production) is increased at least two-fold, preferably ten-fold, most preferably thirty-fold.

The term "modulate an immune response" includes inducing (increasing or eliciting) an immune response; and reducing (suppressing) an immune response. An

immunomodulatory method (or protocol) is one that modulates an immune response in a subject.

The term "therapeutically effective" applied to dose or amount refers to that quantity of a compound or pharmaceutical composition or vaccine that is sufficient to result in a desired activity upon administration to a mammal in need thereof. As used herein with respect to pharmaceutical compositions or vaccines, the term "therapeutically effective amount/dose" is used interchangeably with the term "immunogenically effective amount/dose" and refers to the amount/dose of a compound (*e.g.*, an epitope presented as part of the construct of the invention) or pharmaceutical composition or vaccine that is sufficient to produce an effective immune response upon administration to a mammal.

As used herein, the term "cytokine" refers to any one of the numerous factors that exert a variety of effects on cells, for example, inducing growth or proliferation. Non-limiting examples of cytokines which may be used alone or in combination in the practice of the present invention include, interleukin-2 (IL-2), stem cell factor (SCF), interleukin 3, colony stimulating factor (GM-CSF), interleukin-1 alpha (IL-1I), interleukin-11 (IL-11), MIP-11, leukemia inhibitory factor (LIF), c-kit ligand, thrombopoietin (TPO) and flt3 ligand. The present invention also includes culture conditions in which one or more cytokine is specifically excluded from the medium. Cytokines are commercially available from several vendors such as, for example, Genzyme (Framingham, MA), Genetech (South San Francisco, CA), Amgen (Thousand Oaks, CA), R&D Systems (Minneapolis, MN) and Immunex (Seattle, WA). It is intended, although not always explicitly stated, that molecules having similar biological activity as wild-type or purified cytokines (*e.g.*, recombinantly produced or muteins thereof) are intended to be used within the spirit and scope of the invention.

The term "subject" as used herein refers to an animal having an immune system, preferably a mammal (*e.g.*, rodent such as mouse). In particular, the term refers to humans.

The term "about" or "approximately" usually means within 20%, more preferably within 10%, and most preferably still within 5% of a given value or range. Alternatively, especially in biological systems (*e.g.*, when measuring an immune response), the term "about" means within about a log (*i.e.*, an order of magnitude)

preferably within a factor of two of a given value.

## Molecular Biology Related Definitions

The term "isolated" means separated from constituents, cellular and otherwise, in which the nucleic acid molecule, peptide, polypeptide, protein, or fragments thereof, are normally associated with in nature. For example, with respect to a nucleic acid molecule, an isolated nucleic acid is one that is separated from the 5' and 3' sequences with which it is normally associated in the chromosome. As is apparent to those of skill in the art, a non-naturally occurring polynucleotide, peptide, polypeptide, protein, or fragments thereof, does not require "isolation" to distinguish it from its naturally occurring counterpart. In addition, a "concentrated", "separated" or "diluted" nucleic acid molecule, peptide, polypeptide, protein, or fragments thereof, is distinguishable from its naturally occurring counterpart in that the concentration or number of molecules per volume is greater than "concentrated" or less than "separated" than that of its naturally occurring counterpart. A nucleic acid molecule, peptide, polypeptide, protein, or fragments thereof, which differs from the naturally occurring counterpart in its primary sequence or for example, by its glycosylation pattern, need not be present in its isolated form since it is distinguishable from its naturally occurring counterpart by its primary sequence, or alternatively, by another characteristic such as glycosylation pattern. Although not explicitly stated for each of the inventions disclosed herein, it is to be understood that all of the above embodiments for each of the compositions disclosed below and under the appropriate conditions, are provided by this invention. Thus, a non-naturally occurring nucleic acid is provided as a separate embodiment from the isolated naturally occurring nucleic acid. A protein produced in a bacterial cell is provided as a separate embodiment from the naturally occurring protein isolated from a eukaryotic cell in which it is produced in nature.

"coding sequence" or a sequence "encoding" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in the production of that RNA, polypeptide, protein, or enzyme, *i.e.*, the nucleotide sequence encodes an amino acid sequence for that polypeptide, protein or enzyme. A coding sequence for a protein may include a start codon (usually ATG) and a

stop codon.

The term "gene", also called a "structural gene" means a DNA sequence that codes for or corresponds to a particular sequence of amino acids which comprise all or part of one or more proteins, and may or may not include regulatory DNA sequences, such as promoter sequences, that determine for example the conditions under which the gene is expressed. The transcribed region of a gene can include 5'- and 3'-untranslated regions (UTRs) and introns in addition to the translated (coding) region.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A coding sequence is "under the control" of or "operably (or operatively) associated with" transcriptional and translational control sequences in a cell. RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced (if it contains introns) and translated into the protein encoded by the coding sequence. "Operatively associated" refers to a juxtaposition wherein the elements are in an arrangement allowing them to function in concert.

The terms "express" and "expression" mean allowing or causing the information in a gene or DNA sequence to become manifest, for example producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene or DNA sequence. A DNA sequence is expressed in or by a cell to form an "expression product" such as a mRNA or a protein. The expression product itself, *e.g.* the resulting mRNA or protein, may also be said to be "expressed" by the cell. An expression product can be characterized as intracellular, extracellular or secreted. The term "intracellular" means something that is inside a cell. The term "extracellular" means something that is outside a cell. A substance is "secreted" by a cell if it appears in

significant measure outside the cell, from somewhere on or inside the cell. "Conditions that permit expression" *in vitro* are culture conditions of temperature (generally about 37°C), humidity (humid atmosphere), carbon dioxide concentration to maintain pH (generally about 5% $CO_2$ to about 15% $CO_2$), pH (generally about 7.0 to 8.0, preferably 7.5), and culture fluid components that depend on host cell type. *In vivo*, the conditions that permit expression are primarily the health of the non-human transgenic animal, which depends on adequate nutrition, water, habitation, and environmental conditions (light-dark cycle, temperature, humidity, noise level). In either system, expression may depend on a repressor or inducer control system, as well known in the art.

The term "heterologous" refers to a combination of elements not naturally occurring in a particular locus. For example, heterologous DNA refers to DNA not naturally located in the cell or in a particular chromosomal site of the cell. A heterologous expression regulatory element is such an element operatively associated with a different gene than the one it is operatively associated with in nature. In the context of the present invention, a construct coding sequence is heterologous to the vector DNA in which it is inserted for cloning or expression and it is heterologous to a host cell containing such a vector in which it is expressed, *e.g.*, a CHO cell.

The term "gene delivery", "gene transfer", "transfection", and the like as used herein mean the introduction of a "foreign" (*i.e.*, extrinsic or extracellular) gene, DNA or RNA sequence into a host cell, so that the host cell will express the introduced gene or sequence to produce a desired substance, typically a protein or enzyme encoded by the introduced gene or sequence. The introduced gene or sequence may also be called a "cloned" or "foreign" gene or sequence, may include regulatory or control sequences, such as start, stop, promoter, signal, secretion, or other sequences used by a cell's genetic machinery. The gene or sequence may include nonfunctional sequences or sequences with no known function. A host cell that receives and expresses introduced DNA or RNA has been "transfected" and is a "transfectant" or a "clone." The DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell, or cells of a different genus or species.

A "gene delivery vehicle" is defined as any molecule that can carry inserted polynucleotides into a host cell. Examples of gene delivery vehicles are

liposomes, biocompatible polymers, including natural polymers and synthetic polymers; lipoproteins; polypeptides; polysaccharides; lipopolysaccharides; artificial viral envelopes; metal particles; and bacteria, viruses, such as baculovirus, adenovirus, retrovirus, lentivirus, and adeno-associated virus, bacteriophage, cosmid, plasmid, fungal vectors and other recombination vehicles typically used in the art, which have been described for expression in a variety of eukaryotic and prokaryotic hosts, and may be used for gene therapy as well as for simple protein expression.

The terms "vector", "cloning vector" and "expression vector" mean the gene delivery vehicle by which a DNA or RNA sequence (*e.g.*, a foreign gene) can be introduced into a host cell, so as to transform the host and promote expression (*e.g.*, transcription and translation) of the introduced sequence.

Vectors typically comprise the DNA of a transmissible agent, into which foreign DNA is inserted. A common way to insert one segment of DNA into another segment of DNA involves the use of enzymes called restriction enzymes that cleave DNA at specific sites (specific groups of nucleotides) called restriction sites. A "cassette" refers to a DNA segment that can be inserted into a vector or into another piece of DNA at a defined restriction site. Preferably, a cassette is an "expression cassette" in which the DNA is a coding sequence or segment of DNA that codes for an expression product that can be inserted into a vector at defined restriction sites. The cassette restriction sites generally are designed to ensure insertion of the cassette in the proper reading frame. Generally, foreign DNA is inserted at one or more restriction sites of the vector DNA, and then is carried by the vector into a host cell along with the transmissible vector DNA. A segment or sequence of DNA having inserted or added DNA, such as an expression vector, can also be called a "DNA construct." A common type of vector is a "plasmid" that generally is a self-contained molecule of double-stranded DNA, usually of bacterial origin, that can readily accept additional (foreign) DNA and which can be readily introduced into a suitable host cell. A plasmid vector often contains coding DNA and promoter DNA and has one or more restriction sites suitable for inserting foreign DNA. A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of eukaryotic and prokaryotic hosts. Non-limiting examples include pKK plasmids (Amersham Pharmacia Biotech),

pUC plasmids, pET plasmids (Novagen, Inc., Madison, WI), pRSET or pREP plasmids (Invitrogen, San Diego, CA), or pMAL plasmids (New England Biolabs, Beverly, MA), and many appropriate host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. Recombinant cloning vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, *e.g.* antibiotic resistance, and one or more expression cassettes.

The term "host cell" or "recipient cell" means any cell of any organism that is selected, modified, transformed, grown, or used or manipulated in any way by introduction of a nucleic acid or vector encoding a variant construct, for the production of a substance by the cell, for example the expression by the cell of a gene, a DNA or RNA sequence, or protein, *i.e.*, the variant construct. The host cell may be found *in vitro*, *i.e.*, in tissue culture, or *in vivo*, *i.e.*, in a microbe, plant or animal. These terms are intended to include progeny of a single cell, and progeny may not necessarily be completely identical (in morphology or in genomic or total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation or introduction of further variations. The cells may be prokaryotic or eukaryotic, and include, but are not limited to, bacterial cells, yeast cells, animal cells, and mammalian cells, *e.g.*, murine, rat, hamster, simian, or human cells.

The term "expression system" means a host cell and compatible vector under suitable conditions, *e.g.*, for the expression of a protein coded for by foreign DNA carried by the vector and introduced to the host cell. Preferably the introduced nucleic acid is stably or transiently maintained in the host cell. Stable maintenance typically requires that the introduced nucleic acid either contains an origin of replication compatible with the host cell or integrates into a replicon of the host cell such as an extrachromosoml replicon (*e.g.*, a plasmid) or a nuclear or mitochondrial chromosome. Common expression systems include *E. coli* host cells and plasmid vectors, insect host cells and *Baculovirus* vectors, and mammalian host cells and vectors. In a specific embodiment, the construct is expressed in COS-1 or CHO cells. Other suitable cells include NSO cells, HeLa cells, 293e (human kidney cells), mouse primary myoblasts and NIH 3T3 cells.

The term "culturing" refers to the *in vitro* propagation of host cells or

organisms on or in media of various kinds. Preferably, culturing occurs under conditions that permit expression of the variant construct. By "expanded" is meant any proliferation or division of cells.

The terms "mutant" and "mutation" mean any detectable change in genetic material, *e.g.*, DNA, or any process, mechanism, or result of such a change. This includes gene mutations, in which the structure (*e.g.*, DNA sequence) of a gene is altered, any gene or DNA arising from any mutation process, and any expression product (*e.g.*, protein or enzyme) expressed by a modified gene or DNA sequence. The term "variant" may also be used to indicate a modified or altered gene, DNA sequence, enzyme, cell, etc., *i.e.*, any kind of mutant.

As used herein, the term "oligonucleotide" refers to a nucleic acid, generally of at least 10, preferably at least 15, and more preferably at least 20 nucleotides, preferably no more than about 100 nucleotides, that is hybridizable to a genomic DNA molecule, a cDNA molecule, or an mRNA molecule having a sequence of interest. Oligonucleotides can be labeled, *e.g.*, with $^{32}$P-nucleotides or nucleotides to which a label, such as biotin, has been covalently conjugated. In one embodiment, a labeled oligonucleotide can be used as a probe to detect the presence of a nucleic acid. In another embodiment, oligonucleotides (one or both of which may be labeled) can be used as PCR primers, either for cloning full length or a fragment of the epitope string, or to detect the presence of nucleic acids encoding the epitope string. In a further embodiment, an oligonucleotide of the invention can form a triple helix with a epitope string-encoding DNA molecule, *e.g.*, for purification purposes. Generally, oligonucleotides are prepared synthetically, preferably on a nucleic acid synthesizer. Accordingly, oligonucleotides can be prepared with non-naturally occurring phosphoester analog bonds, such as thioester bonds, etc.

To induce an immune response in a subject, the peptide constructs of the invention can be administered as polynucleotides encoding the polypeptides. The polynucleotides can be administered in a gene delivery vehicle or by inserting into a host cell which in turn recombinantly transcribes, translates and processes the encoded polypeptide. Isolated host cells containing the polynucleotides of this invention in a pharmaceutically acceptable carrier can be therefore combined with appropriate and

effective amounts of an adjuvant, cytokine or co-stimulatory molecule for an effective vaccine regimen. In one embodiment, the host cell is an APC such as a dendritic cell (DC), a monocyte/macrophage, a B lymphocyte, or other cell type(s) expressing the necessary MHC/co-stimulatory molecules. The host cell can be further modified by inserting a polynucleotide coding for an effective amount of either or both a cytokine and a co-stimulatory molecule.

APCs can be transduced *in vitro* with viral vectors encoding the peptide constructs of the invention. The most common viral vectors include recombinant poxviruses such as vaccinia and fowlpox virus (Bronte, *et al.* (1997) PNAS 94:3183-3188; Kim, *et al.* (1997) J. Immunother. 20:276-286) and, preferentially, adenovirus (Arthur, *et al.* (1997) J. Immunol. 159:1393-1403; Wan, *et al.* (1997) Human Gene Therapy 8:1355-1363; Huang, *et al.* (1995) J. Virol. 69:2257-2263). Retrovirus also may be used for transduction (Marin, *et al.* (1996) J. Virol. 70:2957-2962). Transduced APCs can subsequently be administered to the host via an intravenous, subcutaneous, intranasal, intramuscular or intraperitoneal route of delivery. In a further embodiment, APCs or immune effector cells are administered with an effective amount of a stimulatory cytokine, such as IL-2 or a co-stimulatory molecule.

Alternatively, *in vivo* transduction of DCs, or other APCs, can be accomplished by administration of viral vectors via different routes including intravenous, intramuscular, intranasal, intraperitoneal or cutaneous delivery. The preferred method is cutaneous delivery of Ad vector at multiple sites using a total dose of approximately $1x10^{10}$-$1x10^{12}$ i.u.

Although viral gene delivery is more efficient, DCs can also be transduced *in vitro/ex vivo* by non-viral gene delivery methods such as electroporation, calcium phosphate precipitation or cationic lipid/plasmid DNA complexes (Arthur *et al.* (1997) Cancer Gene Therapy 4:17-25). Gene gun delivery or injection of naked plasmid DNA into the skin also leads to transduction of DCs (Condon *et al.* (1996) Nature Med. 2:1122-1128; Raz et al (1994) PNAS 91:9519-9523). Intramuscular delivery of plasmid DNA may also be used for immunization (Rosato *et al.* (1997) Human Gene Therapy 8:1451-1458).

**Examples of Useful APC Receptors Targeted by the Constructs of the Invention**

As specified above, the peptide constructs of the invention comprise APC-targeting sequences.

Typically, antigen presenting cells (APCs) present exogenous antigen on major histocompatibility complex class II (MHC) molecules, and endogenously synthesized antigen on MHC I molecules. In exceptional circumstances exogenous antigens can be presented on MHC I molecules; this phenomenon is central to indirect presentation of antigens, particularly as it relates to priming of naïve CD8+ T lymphocytes specific for tumors and other noninfectious agents (Bevan, J. Exp. Med. 182, 639-641 (1995)).

To generate a primary CTL response to tumor-associated antigens (TAAs), with the capacity to eliminate tumor cells, APCs have to present peptides complexed to MHC class I. It is now becoming apparent that professional APCs, DCs, monocytes and macrophages are capable of internalizing exogenous antigens for processing and presentation on MHC class I molecules. Several mechanisms by which APCs may incorporate peptides and present peptides in association with MHC class I have been reported. Firstly, peptides from degraded proteins may be produced in high concentrations externally and then exchanged with peptides on the surface MHC class I molecules (Carbone and Bevan (1990) J. Exp. Med. 171: 377-387; Pfeifer *et al.* (1993) Nature 361: 359-362). Alternatively, APCs may take up antigen by endocytosis or phagocytosis, process it by proteolysis in the endosomal vesicle where it associates with MHC class I and then it is transported to the surface without having ever entered the cytosol (Bachmann *et al.* (1995) Eur. J. Immunol. 25: 1739-1743; Liu *et al.* (1997) Scand. J. Immunol. 45: 527-533). Recently, a third mechanism whereby exogenous antigens were internalized by APCs, transferred to the cytosol, processed and then presented via the classical endogenous MHC class I pathway was demonstrated for both macrophages (Kovacsovics-Bankowski and Rock, (1995) Science 267: 243-246) and DCs (Rodriguez *et al.*, (1999) Nature Cell Biol. 1: 362-368).

It has been demonstrated previously that exogenous antigens chaperoned by heat shock proteins (HSP) such as gp96 can be taken up by APCs and presented through their MHC I molecules, leading to stimulation of antigen-specific CD8+ T cells (Suto and Srivastava, Science 269, 1585-1588 (1995)). A wide array of peptides are chaperoned by gp96, depending upon the source from which they are isolated (Srivastava *et al.*, Immunity 8, 657-665 (1998)). Tumor-derived gp96 carries tumor-antigenic peptides (Ishii *et al.*, J. Immunol. 162, 1303-1309 (1999)), gp96 preparations from virus-infected cells carry viral epitopes (Suto and Srivastava, Science 269, 1585-1588 (1995); Nieland *et al.*, Proc. Natl Acad. Sci. USA 95, 1800-1805 (1998)) and gp96 preparations from cells transfected with model antigens such as ovalbumin or -galactosidase are associated with the corresponding epitopes (Arnold *et al.*, J. Exp. Med. 182, 885-889 (1995); Breloer *et al.*, Eur. J. Immunol. 28, 1016-1021 (1998)). The association of gp96 with peptides occurs *in vivo* (Ménoret and Srivastava, Biochem. Biophys. Res. Commun. 262, 813-818 (1999)). Complexes of gp96 with peptides, whether isolated from cells (Tamura *et al.*, Science 278, 117-120 (1997)) or reconstituted *in vitro* (Blachere *et al.*, J. Exp. Med. 186, 1183-1406 (1997)), are excellent immunogens and have been used extensively to elicit CD8+ T cell responses specific to the gp96-chaperoned antigenic peptides.

Binder *et al.* (Nature Immunol. (2000) 1:151-155) have shown that APCs can take up exogenous antigenic peptides chaperoned by heat shock protein gp96 and re-present them through the endogenous pathway on their MHC class I molecules. The high efficiency of this process has been attributed previously to a receptor for gp96 on APCs. The CD91 molecule (also called 2-macroglobulin receptor or the low density lipoprotein-related protein) was suggested by Binder *et al.* to be a cell surface receptor for the heat shock protein gp96. CD91 is present on cells of monocytic lineage as well as hepatocytes, fibroblasts and keratinocytes. Binder *et al.* have shown that HSP gp96 is a ligand for CD91. The p80 fragment of CD91 shown to bind gp96 directly is an N-terminal degradation product of the CD91 $\alpha$ subunit. APC-gp96 interaction leads to interaction of gp96 with CD91 and consequent re-presentation of gp96-chaperoned peptides by MHC I molecules of the APC, followed by stimulation of antigen-specific T cells (Suto and Srivastava, Science 269, 1585-1588 (1995)) and secretion of

proinflammatory cytokines such as TNF, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 12 (Basu *et al.*, Int. Immunol. (2000) 12:1539-1546).

Similarly, Suzie *et al.* (Proc. Natl. Acad. Sci. USA, 94:13146-13151 (1997)) have shown that a soluble hsp70 fusion protein having a large fragment of chicken ovalbumin as fusion partner could, in the absence of adjuvants, stimulate H-$2^b$ mice to produce ovalbumin-specific CD8+ CTL. Mice immunized with heat shock proteins (hsps) isolated from mouse tumor cells (donor cells) produce CD8+ cytotoxic T lymphocytes (CTL) that recognize donor cell peptides in association with the major histocompatibility complex (MHC) class I proteins of the responding mouse.

It follows that the hsp molecules are capable of delivering non-covalently associated peptides to MHC class I proteins of other (recipient) cells, including APCs. Accordingly, in one of the embodiments of the present invention, the APC targeting sequence is derived from an hsp sequence, which mediates interaction with the hsp receptor (HSR) on the surface of APC (*e.g.*, CD91 receptor).

DCs internalize exogenous antigens by fluid-phase pinocytosis or by receptor-mediated endocytosis. DCs express several receptors that facilitate the internalization and presentation of antigens, including C-type lectin receptors such as the mannose receptor (Sallusto *et al.*, (1995) J. Exp. Med. 182:389), DEC205 (Jiang *et al.*, (1995) Nature 375:151) and DC-SIGN (Engering *et al.*, J Immunol (2002) 168:2118-2126), as well as receptors for the Fc domain of Igs (Sallusto and Lanzavecchia (1994) J. Exp. Med. 179:1109).

FcRs are expressed on most cells of the hemopoietic lineages, including DCs, and play a pivotal role in linking the humoral and cellular arms of the immune response. Antigen-IgG complexes (immune complexes (ICs)), which bind to and cross-link FcRs, mediate a variety of responses *in vitro* ranging from phagocytosis to antibody-mediated cellular cytotoxicity. FcR-mediated internalization of ICs by DCs is associated with enhanced presentation of both MHC class I- and MHC class II-binding peptides derived from the antigen present in the ICs and leads to activation of the DCs and enables DCs to prime peptide-specific $CD8^+$ CTLs *in vivo* (Amigorena and Bonnerot (1999)

Semin. Immunol. 11:385; Amigorena and Bonnerot (1999) Immunol. Rev. 172:279; Regnault *et al.* (1999) J. Exp. Med. 189:371).

Schuurhuis *et al.* (J Immunol (2002) 168:2240-2246) have shown that OVA/anti-OVA IC-treated DCs primed CTLs against the dominant CTL epitope derived from the OVA Ag present in the ICs.

Among the more than eight members of the IgG Fc receptor family, one receptor, FcγRI (CD64), is constitutively expressed only on monocytes, macrophages and DCs (Pan *et al.* (1990) J. Immunol. 145: 267-275; Fanger *et al.* (1997) J. Immunol. 158: 3090-3098). Targeting antigen to FcγRI on DC in the form of ICs was shown to result in efficient class I-restricted presentation that required proteosomal degradation and was dependent on functional TAP (transporter associated with antigen processing) (Regnault *et al.* (1999) J. Exp. Med., 189: 371-380). Kaufman *et al.* ((1996) Tumor Target. 2: 17-28) have shown in human clinical trials that a bispecific antibody specific for FcγRI efficiently targets monocytes and induces a host anti-tumor response in some patients. Furthermore, Wallace *et al.* ((2001) J. Immunol. Methods 248: 183-194) have recently demonstrated that a fusion protein based on a monoclonal antibody (mAb) that targets FcγRI, in which heavy chain CH2 and CH3 domains were removed and replaced with the prostate specific antigen (PSA), was internalized and processed by the human myeloid THP-1 cell line resulting in presentation of MHC class I-associated PSA peptides and lysis of THP-1 by PSA-specific human CTL.

It follows that the IgG-based complexes (*e.g.*, immune complexes (ICs) or chimeric IgG fusion polypeptides), which bind to FcRs are capable of delivering non-covalently associated peptides to MHC class I proteins of other (recipient) cells, including APCs. Accordingly, in one of the embodiments of the present invention, the APC targeting sequence is derived from an IgG sequence, which mediates interaction with the FcR on the surface of APC (*e.g.*, FcγRI).

Several receptors expressed by immature DCs belong to the C-type lectin superfamily, including Langerin (CD207), the mannose receptor (MR; CD206), and DEC-205 (CD205) (Mason, ed. In *Leucocyte Typing Vol. VII*: 2000 Oxford University Press, Oxford).

Mucin (MUC1) is highly expressed in adenocarcinomas. In mice, oxidized mannan linked to MUC1 (M-FP), given *in vivo*, induces potent MHC-restricted CTL and tumor protection. Apostolopoulos *et al.* (Vaccine (2000) 18: 3174-84) have shown that murine mannose receptor (MR) bearing macrophages derived from peritoneal exudate cells (PEC) and cultured *ex vivo* with M-FP can, after adoptive transfer, efficiently present MUC1 to T cells, leading to the generation of high frequency of CTL and protection from tumor challenge. M-FP targets the MR and ensures rapid passage of peptides to MHC class I molecules, and can also directly stimulate *in vitro* IL-12 production by macrophages. In addition, targeting MR in other studies was shown to lead to efficient class II presentation, as after binding to the MR there is internalization with passage to lysosomes and phagosomes (Tan *et al.* (1997) Eur. J. Immunol. 27: 2426-2432; Engering *et al.* (1997) Eur. J. Immunol. 27: 2417-2425; Wileman (1985) J. Biochem. 260: 7387-7393; Prigozy (1997) Immunity 6: 187-193).

Mahnke *et al.* (J Cell Biol (2000) 151:673-84) have demonstrated in DCs that the DEC-205 multilectin receptor targets late endosomes or lysosomes rich in MHC class II products, whereas the homologous macrophage mannose receptor (MMR) is found in more peripheral endosomes. It was concluded that the DEC-205 cytosolic domain mediates a new pathway of receptor-mediated endocytosis that entails efficient recycling through late endosomes and a greatly enhanced efficiency of antigen presentation to $CD4^+$ T cells.

Geijtenbeek *et al.* ((2000) Cell 100:575) have recently identified a novel C-type lectin, DC-specific ICAM-grabbing non-integrin (DC-SIGN; CD209), that is exclusively expressed on DCs, in contrast to the MR and DEC-205, which are also expressed on other cell types. DC-SIGN functions as cell adhesion receptor mediating both DC migration and T cell activation. DC-SIGN also functions as an HIV-1R that captures HIVgp120 and facilitates DC-induced HIV transmission of T cells. Internalization motifs in the cytoplasmic tail of DC-SIGN hint to a function of DC-SIGN as endocytic receptor. Engering *et al.* (J Immunol (2002) 168:2118-2126) have demonstrated that on DCs DC-SIGN is rapidly internalized upon binding of soluble ligand and are targeted to late endosomes/lysosomes. Moreover, ligands internalized by DC-SIGN are efficiently processed and presented to CD4+ T cells.

It follows that at least some of C-type lectin receptors on the surface of APCs (such as mannose receptor (MR; CD206), DEC-205 (CD205) and DC-SIGN (CD209)) have the potency to be used for targeting antigens (*e.g.*, TAAs) specifically to APCs (*e.g.*, DCs) to induce antigen-specific immunity. Accordingly, in a specific embodiment, the APC targeting sequence of the invention comprises the sequence targeting C-type lectin receptors.

## Nucleic Acids and Gene Vaccines

Recombinant nucleic acids, particularly DNA molecules, provide for efficient expression of the peptide constructs of the invention. In one embodiment, the invention provides a nucleic acid molecule encoding the APC-targeted peptide construct described above. The invention also provides an expression vector comprising such nucleic acid operably associated with an expression control sequence(s) and a host cell (*e.g.*, APC transfected or transformed with the expression vector) and a recombinant non-human host comprising such nucleic acid. According to a specific embodiment, the expression vector comprising the nucleic acid encoding the epitope constructs of the invention may further comprise or may be combined with an APC targeting mechanism and may therefore be targeted directly to be expressed in APCs of the host. The peptide construct of the invention can be produced recombinantly by isolating it from the host cells grown under conditions that permit expression of the nucleic acid encoding it.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual,* Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook *et al.*, 1989"); *DNA Cloning: A Practical Approach,* Volumes I and II (Glover ed. 1985); *Oligonucleotide Synthesis* (Gait ed. 1984); *Nucleic Acid Hybridization*, Hames & Higgins eds. (1985); *Transcription And Translation,* Hames & Higgins, eds. (1984); *Animal Cell Culture,* Freshney, ed. (1986); *Immobilized Cells And Enzymes,* IRL Press, (1986); Perbal, *A Practical Guide To Molecular Cloning* (1984); Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc.

(1994); Goeddel *et al.*, *Gene Expression Technology*, Academic Press (1991); Gacesa and Ramji, *Vectors: Essential Data Series*, John Wiley & Sons (1994).

In a specific embodiment, vectors comprising the nucleotide sequence encoding the peptide construct of the invention are administered to treat or prevent a disease or disorder associated with the expression or function of a molecule to which the construct elicits a specific immune response.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, *see*, U. S. Patent Nos. 6,228,844; 5,693,622; 5,589,466; 5,580,859; 6,214,804; and 5,703,055, International Patent Publication Nos. WO 90/11092, WO 89/12458, WO 94/29469, EP 1026253, Goldspiel *et al.*, Clinical Pharmacy 1993, 12:488-505; Wu and Wu, Biotherapy 1991, 3:87-95; Tolstoshev, Ann. Rev. Pharmacol. Toxicol. 1993, 32:573-596; Mulligan, Science 1993, 260:926-932; and Morgan and Anderson, Ann. Rev. Biochem. 1993, 62:191-217; and May, TIBTECH 1993, 11:155-215. Methods commonly known in the art of recombinant DNA technology that can be used are described in Ausubel *et al.*, (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY; and in Chapters 12 and 13, Dracopoli *et al.*, (eds.), 1994, Current Protocols in Human Genetics, John Wiley & Sons, NY. Vectors suitable for gene therapy are described above.

The present invention also provides delivery vehicles suitable for delivery of a nucleic acid molecule of the invention into cells (whether *in vivo*, *ex vivo*, or *in vitro*). The nucleic acid molecule of the invention can be contained within a cloning or expression vector. These vectors (especially expression vectors) can in turn be manipulated to assume any of a number of forms which may, for example, facilitate delivery to and/or entry into a cell.

When the vectors are used for gene therapy *in vivo* or *ex vivo*, a pharmaceutically acceptable vector is preferred, such as a replication-incompetent retroviral or adenoviral vector. Pharmaceutically acceptable vectors containing the nucleic acids of this invention can be further modified for transient or stable expression of the inserted polynucleotide.

Host cells containing the nucleic acids of the invention are useful for the recombinant replication of the polynucleotides and for the recombinant production of peptides. Alternatively, the cells may be used to induce an immune response in a subject in the methods described herein. When the host cells are APCs, they can be used to expand a population of immune effector cells such as tumor infiltrating lymphocytes (TILs), which in turn are useful in adoptive immunotherapies.

In one aspect, the therapeutic vector comprises a nucleic acid molecule that expresses the construct in a suitable host. In particular, such a vector has a promoter operably linked to the coding sequence for the peptide epitope construct. The promoter can be inducible or constitutive and, optionally, tissue-specific. In another embodiment, a nucleic acid molecule is used in which the antibody coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for expression of the construct from a nucleic acid molecule that has integrated into the genome (Koller and Smithies, Proc. Natl. Acad. Sci. USA 1989, 86:8932-8935; Zijlstra *et al.*, Nature 1989, 342:435-438).

Delivery of the vector into a patient may be either direct, in which case the patient is directly exposed to the vector or a delivery complex, or indirect, in which case, cells are first transformed with the vector *in vitro* then transplanted into the patient. These two approaches are known, respectively, as *in vivo* and *ex vivo* gene therapy.

In a specific embodiment, the vector is directly administered *in vivo,* where it enters the cells of the organism and mediates expression of the constructs. This can be accomplished by any of numerous methods known in the art, *e.g.*, by constructing it as part of an appropriate expression vector and administering it so that it becomes intracellular, *e.g.*, by infection using a defective or attenuated retroviral or other viral vector (*see*, U.S. Patent No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont); or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in biopolymers (*e.g.*, poly-β1→4-N-acetylglucosamine polysaccharide; *see*, U.S. Patent No. 5,635,493), encapsulation in liposomes, microparticles, or microcapsules; by administering it in linkage to a peptide or other ligand known to enter the nucleus; or by administering it in linkage to a ligand subject to receptor-mediated endocytosis (*see*, *e.g.*, Wu and Wu, J.

Biol. Chem. 1987, 62:4429-4432), etc. In another embodiment, a nucleic acid/ligand complex can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, *e.g.*, PCT Publication Nos. WO 92/06180, WO 92/22635, WO 92/20316 and WO 93/14188). These methods are in addition to those discussed above in conjunction with "Viral and Non-viral Vectors".

The form and amount of therapeutic nucleic acid envisioned for use depends on the type of disease and the severity of the desired effect, patient state, etc., and can be determined by one skilled in the art.

## Therapeutic Use of the Constructs of the Invention

The invention also provides methods for treating a disease by administration of a therapeutic of the invention. Such therapeutics include the peptide constructs of the invention and nucleic acids encoding the constructs of the invention.

In the disclosed compositions, the peptide constructs of the invention or the nucleic acids encoding the constructs of the invention are present in immunogenically effective amount. For each specific antigen, the immunogenically effective amount is readily determined experimentally (taking into consideration specific characteristics of a given patient and/or type of treatment) using well-known methods. Generally, this amount is in the range of 0.1μg-100mg of an antigen per kg of the body weight.

The subjects to which the present invention is applicable may be any mammalian or vertebrate species that include, but are not limited to, cows, horses, sheep, pigs, fowl (*e.g.*, chickens), goats, cats, dogs, hamsters, mice, rats, monkeys, rabbits, chimpanzees, and humans. In a preferred embodiment, the subject is a human.

Immunogenicity enhancing methods of the invention can be used to combat various cancers, which include without limitation fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendothelio-sarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, lymphoma, leukemia,

squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, hepatocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinoma, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, and retinoblastoma, among others.

Methods of the invention are also useful in treatment of infections, which include, but are not limited to, parasitic infections (such as those caused by plasmodial species, etc.), viral infections (such as those caused by influenza viruses, leukemia viruses, immunodeficiency viruses such as HIV, papilloma viruses, herpes virus, hepatitis viruses, measles virus, poxviruses, mumps virus, cytomegalovirus [CMV], Epstein-Barr virus, etc.), bacterial infections that involve MHC class I (such as those caused by staphylococcus, streptococcus, pneumococcus, *Neisseria gonorrhea*, *Borrelia*, pseudomonas, mycobacteria, *Salmonella*, etc.), and fungal infections (such as those caused by *Candida*, *Trichophyton*, *Ptyrosporum*, etc.).

Methods of the invention are also useful in treatment of autoimmune diseases, which include, but are not limited to, most forms of arthritis, ulcerative colitis, asthma, multiple sclerosis, lupus, and myasthenia gravis.

## *Treatment and Prevention of Cancers*

In a preferred embodiment, the invention provides methods of treating or preventing cancers. The method includes administering to a subject in need of such treatment or prevention a peptide construct of the invention or a nucleic acids encoding it.

Cancers, including any disease or disorder characterized by uncontrolled cell growth, in which the tumor cells express a tumor associated antigen as described herein having immunogenic properties relevant to human cancers, can be treated or prevented by administration of a construct of the invention. Whether a particular therapeutic is effective to treat or prevent a certain type of cancer can be determined by

any method known in the art.

In other embodiments of the invention, the subject being treated with the construct may, optionally, be treated with other cancer treatments such as surgery, radiation therapy, or chemotherapy. In particular, the therapeutic of the invention used to treat or prevent cancer may be administered in conjunction with one or a combination of chemotherapeutic agents including, but not limited to, methotrexate, taxol, taxotere, mercaptopurine, thioguanine, hydroxyurea, cytarabine, cyclophosphamide, ifosfamide, nitrosoureas, cisplatin, carboplatin, mitomycin, dacarbazine, procarbizine, etoposides, campathecins, bleomycin, doxorubicin, idarubicin, daunorubicin, dactinomycin, plicamycin, mitoxantrone, asparaginase, vinblastine, vincristine, vinorelbine, paclitaxel, docetaxel, etc.

## *Vaccine Formulations and Administration*

The invention also provides vaccine formulations containing peptide constructs of the invention or nucleic acids encoding them, which vaccine formulations are suitable for administration to elicit a protective immune (humoral and/or cell mediated) response against cancer cells bearing a ligand as described herein, *e.g.*, for the treatment and prevention of diseases.

The peptide constructs of the invention or nucleic acids encoding them can be used in a variety of formulations, which may vary depending on the intended use.

The peptide constructs of the invention or nucleic acids encoding them can be covalently or non-covalently linked (complexed) to various other molecules, the nature of which may vary depending on the particular purpose. For example, a peptide of the invention can be covalently or non-covalently complexed to a macromolecular carrier, including, but not limited to, natural and synthetic polymers, proteins, polysaccharides, poly(amino acid), polyvinyl alcohol, polyvinyl pyrrolidone, and lipids. A peptide can be conjugated to a fatty acid, for introduction into a liposome. U.S. Pat. No. 5,837,249. A synthetic peptide of the invention can be complexed covalently or non-covalently with a solid support, a variety of which are known in the art. A synthetic antigenic peptide epitope of the invention can be associated with an antigen-presenting matrix with or without co-stimulatory molecules, as described in more detail below.

Examples of protein carriers include, but are not limited to, superantigens, serum albumin, tetanus toxoid, ovalbumin, thyroglobulin, myoglobulin, and immunoglobulin.

Peptide-protein carrier polymers may be formed using conventional crosslinking agents such as carbodiimides. Examples of carbodiimides are 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide (CMC), 1-ethyl-3-(3-dimethyaminopropyl) carbodiimide (EDC) and 1-ethyl-3-(4-azonia-44-dimethylpentyl) carbodiimide.

Examples of other suitable crosslinking agents are cyanogen bromide, glutaraldehyde and succinic anhydride. In general, any of a number of homobifunctional agents including a homobifunctional aldehyde, a homobifunctional epoxide, a homobifinctional imidoester, a homobifinctional N-hydroxysuccinimide ester, a homobifinctional maleimide, a homobifunctional alkyl halide, a homobifunctional pyridyl disulfide, a homobifinctional aryl halide, a homobifimctional hydrazide, a homobi functional diazonium derivative and a homobifinctional photoreactive compound may be used. Also included are heterobifunctional compounds, for example, compounds having an amine-reactive and a sulfhydryl-reactive group, compounds with an amine-reactive and a photoreactive group and compounds with a carbonyl-reactive and a sulfhydryl-reactive group.

Specific examples of such homobifunctional crosslinking agents include the bifunctional N-hydroxysuccinimide esters dithiobis(succinimidylpropionate), disuccinimidyl suberate, and disuccinimidyl tartarate; the bifunctional imidoesters dimethyl adipimidate, dimethyl pimelimidate, and dimethyl suberimidate; the bifunctional sulfhydryl-reactive crosslinkers 1,4-di-[3'-(2'-pyridyldithio) propion-amido]butane, bismaleimidohexane, and bis-N-maleimido-1,8-octane; the bifunctional aryl halides 1,5-difluoro-2,4-dinitrobenzene and 4,4'-difluoro-3,3'-dinitrophenylsulfone; bifunctional photoreactive agents such as bis-[b-(4-azidosalicylamido)ethyl]disulfide; the bifunctional aldehydes formaldehyde, malondialdehyde, succinaldehyde, glutaraldehyde, and adipaldehyde; a bifunctional epoxide such as 1,4-butaneodiol diglycidyl ether, the bifunctional hydrazides adipic acid dihydrazide, carbohydrazide, and succinic acid dihydrazide; the bifunctional diazoniums o-tolidine, diazotized and bis-diazotized benzidine; the bifunctional alkylhalides N1N'-ethylene-bis(iodoacetamide), N1N'-

hexamethylene-bis(iodoacetamide), N1N'-undecamethylene-bis(iodoacetamide), as well as benzylhalides and halomustards, such as ala'-diiodo-p-xylene sulfonic acid and tri(2-chloroethyl)amine, respectively.

Examples of other common heterobifunctional cross-linking agents that may be used to effect the conjugation of proteins to peptides include, but are not limited to, SMCC succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate), MBS (m-maleimidobenzoyl-N-hydroxysuccinimide ester), SIAB (N-succinimidyl(4-iodoacteyl)aminobenzoate), SMPB (succinimidyl-4-(p-maleimidophenyl)butyrate), GMBS (N-(.gamma.-maleimidobutyryloxy)succinimide ester), MPBH (4-(4-N-maleimidopohenyl) butyric acid hydrazide), M2C2H (4-(N-maleimidomethyl) cyclohexane-1-carboxyl-hydrazide), SMPT (succinimidyloxycarbonyl-a-methyl-a-(2-pyridyldithio)toluene), and SPDP(N-succinimidyl 3-(2-pyridyldithio)propionate).

Crosslinking may be accomplished by coupling a carbonyl group to an amine group or to a hydrazide group by reductive amination.

Peptide constructs of the invention also may be formulated as non-covalent attachment of monomers through ionic, adsorptive, or biospecific interactions. Complexes of peptides with highly positively or negatively charged molecules may be done through salt bridge formation under low ionic strength environments, such as in deionized water. Large complexes can be created using charged polymers such as poly-(L-glutamic acid) or poly-(L-lysine) which contain numerous negative and positive charges, respectively. Adsorption of peptides may be done to surfaces such as microparticle latex beads or to other hydrophobic polymers, forming non-covalently associated peptide-superantigen complexes effectively mimicking crosslinked or chemically polymerized protein. Finally, peptides may be non-covalently linked through the use of biospecific interactions between other molecules. For instance, utilization of the strong affinity of biotin for proteins such as avidin or streptavidin or their derivatives could be used to form peptide complexes. These biotin-binding proteins contain four binding sites that can interact with biotin in solution or be covalently attached to another molecule. Wilchek (1988) Anal Biochem. 171:1-32 Peptides can be modified to possess biotin groups using common biotinylation reagents such as the N-hydroxysuccinimidyl ester of D-biotin (NHS-biotin) which reacts with available amine groups on the protein.

Biotinylated peptides then can be incubated with avidin or streptavidin to create large complexes. The molecular mass of such polymers can be regulated through careful control of the molar ratio of biotinylated peptide to avidin or streptavidin.

The peptide constructs of this invention also can be combined with various liquid phase carriers, such as sterile or aqueous solutions, pharmaceutically acceptable carriers, suspensions and emulsions. Examples of non-aqueous solvents include propyl ethylene glycol, polyethylene glycol and vegetable oils. When used to prepare antibodies, the carriers also can include an adjuvant that is useful to non-specifically augment a specific immune response. A skilled artisan can easily determine whether an adjuvant is required and select one. However, for the purpose of illustration only, suitable adjuvants include, but are not limited to, Freund's Complete and Incomplete, mineral salts and polynucleotides.

Suitable preparations of such vaccines and therapeutics include injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, suspension in, liquid prior to injection, may also be prepared. The preparation may also be emulsified, or the constructs antibodies encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, buffered saline, dextrose, glycerol, ethanol, sterile isotonic aqueous buffer or the like and combinations thereof. In addition, if desired, the vaccine preparation may also include minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants that enhance the effectiveness of the vaccine. The construct when prepared as a vaccine can be introduced in microspheres or microcapsules, *e.g.*, prepared from PGLA (*see*, U.S. Patent Nos. 5,814,344, 5,100,669, and 4,849,222; PCT Publication Nos. WO 95/11010 and WO 93/07861).

The effectiveness of an adjuvant may be determined by measuring the induction of an immune response directed against the targeted antigen.

The composition, vaccines, and therapeutics can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. For oral administration, the therapeutics can take the form of, for example, tablets or capsules

prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets can be coated by methods well known in the art. Liquid preparations for oral administration can take the form of, for example, solutions, syrups, emulsions or suspensions, or they can be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations can be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations can also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free concentrate in a sealed container such as a vial or sachette indicating the quantity of active agent. Where the composition is administered by injection, an ampoule of sterile diluent can be provided so that the ingredients may be mixed prior to administration.

In a specific embodiment, the lyophilized construct of the invention is provided in a first container; a second container comprises diluent consisting of an aqueous solution of 50% glycerin, 0.25% phenol, and an antiseptic (*e.g.*, 0.005% brilliant green).

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the vaccine formulations of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

Many methods may be used to introduce the vaccine formulations of the

invention; these include but are not limited to oral, intracerebral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal routes, and via scarification (scratching through the top layers of skin, *e.g.*, using a bifurcated needle) or any other standard routes of immunization.

## Effective Dose and Safety Evaluations

According to the methods of the present invention, the pharmaceutical and vaccine compositions described herein are administered to a patient at immunogenically effective doses, preferably, with minimal toxicity. As recited in the Section entitled "Definitions", "immunogenically effective dose" or "therapeutically effective dose" of disclosed formulations refers to that amount of an antigen-containing composition that is sufficient to produce an effective immune response in the treated subject and therefore sufficient to result in a healthful benefit to said subject.

Following methodologies which are well-established in the art (*see, e.g.*, reports on evaluation of several vaccine formulations in a collaborative effort between the Center for Biological Evaluation and Food and Drug Administration and the National Institute of Allergy and Infectious Diseases [Goldenthal *et al.*, National Cooperative Vaccine Development Working Group. AIDS Res. Hum. Retroviruses, 1993, 9:545-549]), effective doses and toxicity of the compounds and compositions of the instant invention are first determined in preclinical studies using small animal models (*e.g.*, mice) in which these compounds and compositions have been found to be immunogenic and that can be reproducibly immunized by the same route proposed for the human clinical trials.

In a specific embodiment, the efficiency of epitope-specific CD8+ T cell responses to the pharmaceutical and vaccine compositions of the invention is determined by the enzyme-linked immunospot technique (ELISPOT). ELISPOT is a standard method in the art originally developed by the present inventors and their co-workers (Miyahira *et al.*, J. Immunol. Meth., 181: 45-54, 1995) and widely used by others (*see, e.g.*, Guelly *et al.*, Eur. J. Immunol., 32:182-192, 2002; Nikitina and Gabrilovich, Int. J. Cancer, 94:825-833, 2001; Field *et al.*, Immunol. Rev., 182:99-112, 2001; Altfeld *et al.*, J. Immunol., 167:2743-2752, 2001; Skoberne *et al.*, J. Immunol., 167:2209-2218, 2001).

This method employs pairs of antibodies, directed against distinct epitopes of a cytokine, and allows the visualization of cytokine secretion by individual T cells following *in vitro* stimulation with an antigen. ELISPOT has the advantage of detecting only activated/memory T cells and the cytokine release can be detected at the single cell levels, allowing direct determination of T cell frequencies (Czerkinsky *et al.*, J. Immunol. Methods, 25:29, 1988; Taguchi *et al.*, J. Immunol. Methods, 128:65, 1990). The cytokine captured by the immobilized antibody in the ELISPOT assay is detected *in situ* using an insoluble peroxidase substrate. Thus, the cytokine secretion by individual cells is clearly visualized. The high sensitivity and easy performance, allowing a direct enumeration of peptide-reactive T cells without prior *in vitro* expansion, make the ELISPOT assay eminently well suited to monitor and measure T cell responses, particularly, CD8+ T cell responses of very low frequencies. According to alternative embodiments, the efficiency of epitope-specific CD8+ T cell responses to the pharmaceutical and vaccine compositions of the invention can be determined using other art-recognized immunodetection methods such as, *e.g.*, ELISA (Tanguay and Killion, Lymphokine Cytokine Res., 13:259, 1994) and intracellular staining (Carter and Swain, Curr. Opin. Immunol, 9:1977, 1997).

As disclosed herein, for any pharmaceutical composition or vaccine used in the methods of the invention, the therapeutically effective dose can be estimated initially from animal models to achieve a circulating plasma concentration range that includes the $IC_{50}$ (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms). Dose-response curves derived from animal systems are then used to determine testing doses for the initial clinical studies in humans. In safety determinations for each composition, the dose and frequency of immunization should meet or exceed those anticipated for use in the clinical trial.

The dose of peptide constructs, nucleic acids encoding them and other components in the compositions of the present invention is determined to ensure that the dose administered continuously or intermittently will not exceed a certain amount in consideration of the results in test animals and the individual conditions of a patient. A specific dose naturally varies depending on the dosage procedure, the conditions of a patient or a subject animal such as age, body weight, sex, sensitivity, feed, dosage period,

drugs used in combination, seriousness of the disease. The appropriate dose and dosage times under certain conditions can be determined by the test based on the above-described indices and should be decided according to the judgment of the practitioner and each patient's circumstances according to standard clinical techniques. In this connection, the dose of an antigen is generally in the range of 0.1 μg-100 mg per kg of the body weight.

Toxicity and therapeutic efficacy of immunogenic compositions of the invention can be determined by standard pharmaceutical procedures in experimental animals, *e.g.*, by determining the $LD_{50}$ (the dose lethal to 50% of the population) and the $ED_{50}$ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio $LD_{50}/ED_{50}$. Compositions that exhibit large therapeutic indices are preferred. While therapeutics that exhibit toxic side effects can be used (*e.g.*, when treating severe forms of cancer or life-threatening infections), care should be taken to design a delivery system that targets such immunogenic compositions to the specific site (*e.g.*, a tumor or an organ supporting replication of the infectious agent) in order to minimize potential damage to other tissues and organs and, thereby, reduce side effects. As disclosed herein, the constructs of the invention are not only highly immunostimulating at relatively low doses (*e.g.*, 0.1-100 μg per kg of the body weight) but also possess low toxicity and do not produce significant side effects.

As specified above, the data obtained from the animal studies can be used in formulating a range of dosages for use in humans. The therapeutically effective dosage of compositions of the present invention in humans lies preferably within a range of circulating concentrations that include the $ED_{50}$ with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized. Ideally, a single dose should be used.

## EXAMPLE

The following Example illustrates the invention without limiting its scope.

## Example 1: Construction of Epitope String Containing Amino Acid Sequences from CEA

Carcinoembryonic antigen (CEA) is a tumor associated antigen expressed by many types of tumor cells (Thomas *et al.*, Biochem. Biophys. Acta, 1032: 177-79 (1990)). Amino acid sequence of CEA (SEQ ID NO:1; GenBank Accession No. AAA62835) is provided below:

```
MESPSAPPHRWCIPWQRLLLTASLLTFWNPPTTAKLTIESTPFNVAEGKEVLLLVHNLP
QHLFGYSWYKGERVDGNRQIIGYVIGTQQATPGPAYSGREIIYPNASLLIQNIIQNDTG
FYTLHVIKSDLVNEEATGQFRVYPELPKPSISSNNSKPVEDKDAVAFTCEPETQDATYL
WWVNNQSLPVSPRLQLSNGNRTLTLFNVTRNDTASYKCETQNPVSARRSDSVILNVLYG
PDAPTISPLNTSYRSGENLNLSCHAASNPPAQYSWFVNGTFQQSTQELFIPNITVNNSG
SYTCQAHNSDTGLNRTTVTTITVYAEPPKPFITSNNSNPVEDEDAVALTCEPEIQNTTY
LWWVIIRSLPVSPRLQLSNDNRTLTLLSVTRNDVGPYECGIQNELSVDHSDPVILNVLY
GPDDPTISPSYTYYRPGVNLSLSCHAASNPPAQYSWLIDGNIQQHTQELFISNITEKNS
GLYTCQANNSASGHSRTTVKTITVSAELPKPSISSNNSKPVEDKDAVAFTCEPEAQNTT
YLWWVNGQSLPVSPRLQLSNGNRTLTLFNVTRNDARAYVCGIQNSVSANRSDPVTLDVL
YGPDTPIISPPDSSYLSGANLNLSCHSASNPSPQYSWRINGIPQQHTQVLLIAKIQPNN
NGTYACFVSNLATGRNNSIVKSITVSASGTSPGLSAGATAGIMIGVLVGVALI
```

An epitope string containing CTL epitopes of human CEA is constructed with the standard cloning steps and conditions.

The following CEA epitopes are incorporated into epitope string constructs:

| <u>Sequence Type</u> | <u>Sequence</u> |
|---|---|
| CTL | CTL CEA1: YLSGANLNL (SEQ ID NO: 2), Zaremba S., *et al.* (1997) Cancer Research 57(20), 4570-4577. |

| | |
|---|---|
| | CTL CEA2: HLFGYSWYK (SEQ ID NO: 3), Kawashima I., *et al.* (1999) Cancer Research 59(2), 431-435.<br><br>CTL CEA3: IPQQHTQVL (SEQ ID NO: 4), Lu J. and Celis E. (2000) Cancer Research 60(18), 5223-5227. |
| T-helper epitope | HIV gp120, aa 421-444<br>KQIINMWQEVGKAMYAPPISGQIR (SEQ ID NO: 5) |
| B-cell epitope | Amino acids 524-57 of the A3 domain; aa 1-107 of the N-domain of CEA (SEQ ID NO: 1) |

The CEA epitope sequences are then linked to the appropriate flanking sequences and the resulting epitope string construct is linked at the C-terminus to the CD91 APC targeting sequence comprising the gp96 sequence which interacts with the N-terminal p80 fragment of the alpha subunit of CD91, receptor for heat shock proteins (see Binder *et al.*, Nature Immunol. 1: 151-55 (2000)).

The final epitope string construct may be represented by the following general formula:

**N-[Leu-Xaa-Xaa-Asp-Xaa-Xaa-Pro][Xaa-Lys-Xaa-Lys-Phe]-[CEA epitope 1]- [Leu-Xaa-Xaa-Asp-Xaa-Xaa-Pro][Xaa-Lys-Xaa-Lys-Phe]-[CEA epitope 2]-...-[CEA epitope n]-[Leu-Xaa-Xaa-Asp-Xaa-Xaa-Pro][Xaa-Lys-Xaa-Lys-Phe]-[CD91 targeting sequence from gp96]-C**

wherein n represents the number of epitopes and Xaa represents any amino acid.

*   *   *

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended

to fall within the scope of the appended claims.

All patents, applications, publications, test methods, literature, and other materials cited herein are hereby incorporated by reference.